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BIOCHEMISTRY

FOUNDED BY PROF. S. KAKIUCHI

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PREFACE FOR REPUBLICATION

The World War II left blank pages on the record of scientific activities in Japan. This is also the case in biochemistry.

The Journal of Biochemistry, which had been started in 1922 by Dr. S. Kakiuchi, Prof. emeritus of Tokyo University, was compelled to discontinue in 1944 with the final issue of Vol. 36. Since the Journal was widely read, the call for its reissue was intensely raised in Japan as well as abroad. But the difficulties to be surmounted were incalculable for such an undertaking due to the general adverse conditions and the economic one in particular, which prevailed in Japan, in the years subsequent to the end of the hostilities.

Now it is our great pleasure to be able to announce the publication of the Journal of Biochemistry again to be issued by the hands of the Japanese Biochemical Society, under a partial economic aid of the National Science Research Council, Japan. The new issue is No. 1 of Vol. 37 as it follows the serial number of the discontinued Journal.

In conclusion, we should like to recall with profound respect the whole-hearted devotion, both spiritually and economically, of Dr. S. Kakiuchi, who is the founder of this Journal and whose enterprise we are going to follow in every respect, and also, we should like to solicit the kind cooperation of our colleagues and friends all over the world.

Tokyo, May 1951

Editorial Committee

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The Journal of Biochemistry, Vol. 37, No. 1

CHEMICAL STUDIES ON THE SUBSTANCE WHICH IS CONTAMINATED IN THE PAROTID GLAND AND INFLUENCES UPON THE CALCIUM CONTENT OF BLOOD.

By

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(Received for publication, July 13, 1949)

The fact that the parotid gland has some relation upon the calcium metabolism was reported by T. Ogata in the period from 1935 to 1942, and from its experiments he concluded that the substance, secreted from the parotid gland, stimulates the development of borns and teeth. On the relation between the parotid gland and the calcium content of blood, T. Matsuo has experimented and concluded that the venous blood from the parotid gland or the extract of the gland contain the substance which lowers the calcium content of blood and this substance was insoluble in water, but soluble in alcohol, acetone, ether or chloroform and therefore it might be considered as of lipoid nature.

Recently A. Ogata and Z. Ishii have studied on the active substance in the gland and they concluded that the substance was soluble in water and of protein nature. The author has attempted to know the function of the parotid gland concerning the calcium metabolism and at first has studied to detect the chemical nature of the substance, which is contained in the parotid gland and has an influence upon the calcium content of blood.

EXPERIMENTAL

1. Animals and method of a blood calcium measurement

As experimental animals grown-up rabbits which weight about 3 kg. were used. Before the experiment the rabbit was abstained from food for 12 hours. The blood was taken from the ear vein 4 ml. each time and serum was separated by leaving alone in an incubator for one hour. The estimation of the calcium content was done by Kramer and Tisdalls method.

2. *The influence of the successive blood takings upon the calcium content of blood*

To determine whether the change of the calcium content of blood is realy caused by the injection of the extract of the parotid gland or not, it must be examined beforehand that whether the successive blood takings, which are necessary for the calcium estimation, influence upon the calcium content of blood or not. The fastened rabbit for 12 hours was used for this experiment.

The blood was taken two times with an interval of three hours 4 ml. each time, and about them the calcium content was estimated respectively. The results are shown in Table I.

TABLE I

Calcium content of rabbit blood after successive blood takings

No. of rabbit	1		2	
Normal state	17.51 mg.%	Diminution	17.51 mg.%	Diminution
After 4 hours from the first blood taking	16.88	−3.5%	17.10	−2.4%
After 7 hours from the first blood taking	16.88	−3.5	16.88	−3.5

As can be seen from the table, merely by the successive blood takings, the calcium content of blood falls from 2.4 to 3.5 per cent from the original content. Therefore in the experiment when the falls of the calcium content of blood are more than 10 per cent of original value, the changes are considered realy as the influence of the injection of the substance.

3. *The isolation of the substance, which influences upon the calcium content of blood, from the ox parotid gland.*

(A) Water extract of the gland;

The fresh ox parotid gland was freed from fatty tissue and vessels and ground well by a machine.

To 50 g. of this material 100 ml. of physiological salt solution was added and shaken for one hour by a shaking machine. After standing over night in the room temperatue, it was filtrated through the paper and clear solution was obtained.

(B) Alcoholic extract of the gland;

To 50 g. of the ground material 100 ml. of alcohol was added and shaken for one hour by a shaking machine, and the filtrate obtained through filter paper was concentrated at the temperature not exceeding 40° C in the current of carbondioxide under low pressure to syrup.

To this solution five times as much saline solution was added promptly and obtained the emulsion. These solutions were injected to the rabbit subcutaneously each 2 ml. per kilogram of body weight respectively, and 4 and 7 hours after the injections the calcium content of blood was estimated. The results are as follows.

TABLE II

Calcium content of blood after the injection of water or alcohol extract of the parotid gland

Material for an injection	Water extract			
No. of rabbit	3		4	
Before the injection	14.42 mg.%	Diminution	15.08 mg.%	Diminution
4 hours after the injection	12.87	−10.7%	13.42	−10.6%
7 hours after the injection	12.00	−14.2	12.98	−13.5

Material for an injection	Alcohol extract			
No. of rabbit	5		6	
Before the injection	16.48 mg.%	Diminution	17.51 mg.%	Diminution
4 hours after the injection	15.45	−6.3%	16.48	−5.9%
7 hours after the injection	16.27	−1.3	16.48	−5.9

The result show that substance which causes hypocalcaemia in the rabbit proceeds to the water soluble fraction and the alcoholic soluble fraction does not cause any change of the calcium content. From these experiments the substance active as the depriciator of the blood calcium might be considered as the other substance than lipoid.

4. *Preparation of the substance from the parotid gland of an ox*

From the previous experiment we know that the substance which lowers the blood calcium content is other than lipoid like substance. Therefore I tried the preparation of the substance as follows. To 75 g. of the parotid gland ground as before 150 ml. of acetone is added and extracted for one hour, and after standing in the room temperature over night the supernatant liquid is removed. This operation is repeated twice. The acetone solution is reserved for a next experiment. Then the material is extracted with ether completely in the Soxhlet's apparatus. The acetone solution and etheral solution were evaporated to dryness and redissolved in a small amount of alcohol respectively. With these alcoholic solutions the emulsion is prepared by adding physiological salt solution of five times of its volume promptly. In the period from 30 minutes to 1.5 hours after dilution with saline solution, 6 ml. of these emulsions is injected into rabbit respectively and after 5 hours of the injection the estimation of the calcium content of blood was performed. It is known from this experiment that both fractions have no effect upon the calcium content of blood as can be seen from following results.

TABLE III

Calcium content of blood after an injection of acetone soluble fraction

No. of rabbit	7		8	
Before the injection	18.24 mg.%	Diminution	16.95 mg.%	Diminution
5 hours after the injection	18.24	0	16.75	−1.2%

TABLE IV

Calcium content of blood after an injection of ether soluble fraction

No. of rabbit	9		10	
Before the injection	19.38 mg.%	Diminution	19.21 mg.%	Diminution
5 hours after the injection	18.24	−5.8%	18.14	−5.6%

The material, treated with acetone and ether as above, was dried and 10 g. of it was dissolved in 100 ml. of saline solution at pH 8.0 by shaking well. The remainder is removed by a centrifugation. 6 ml. of this solutions is injected into rabbit and examined its effect on the calcium content of blood. The results are shown in Table V.

TABLE V

Calcium content of blood after an injection of water soluble fraction

No. of rabbit	11		12	
Before the experiment	19.05 mg.%	Diminution	16.48 mg.%	Diminution
4 hours after the injection	14.42	−29.5%	12.36	−25.0%
7 hours after the injection	15.45	−18.8	13.90	−15.0

From these experiments it can be said that the substance included in the parotid gland and lowers the blood calciun content is insoluble in organic solvent as alcohol, ether or acetone and soluble in saline solution.

5. *Chemical nature of the active substance.*

To know the chemical nature of the substance I examined the various protein reactions and reduction reactions about the saline solution obtained as above.

TABLE VI

Protein reactions			Reduction reactions
Colour reaction	Sedimentation reaction	Coagulation reaction	Fehling's reaction (−) Nylander's reaction (−)
Biuret reaction (+)	Sulfosalicyl reaction (+)	Heat with acetic acid(+)	
Xanthoprotein reaction (+)	$Pb(C_2H_3O_2)_2$ (+)	Heat with hydrochloric acid (+)	After hydrolyse with acid it shows no reduction reaction.
Millons reaction (+)	$FeCl_3$ (+)		
PbS reaction (+)	$CuSO_4$ (+)		
	$K_4Fe(CN)_6$ (+)		
	Tannic acid (+)		
	Trichloracetic acid (+)		
	Alcohol (+)		

These results indicate that the substance might be considered as of protein nature.

6. *Effect of the proteinase upon the action of the substance.*

To ascertain the protein nature of the active substance I examined whether the activity of the substance is diminished by the digestion with proteinase or not. As the proteinase trypsin (Merck) and pepsin (Sankyo) were used. The enzymes were purified according to Willstaetter's method by adsorption and elusion and removed perfectly the digestive action on lipoid and carbohydrate which were contained as impurity. The enzyme solutions thus obtained indicate the following digestive action against 1% casein solution.

TABLE VII

Test of the purified trypsin and pepsin.

	Sedimentation reaction		Colour reaction
	Sulfosalicyl reaction	Trichlor acetic acid	Biuret reaction
(Casein solution 15 ml. +8% Trypsin solution 5 ml.) (at pH 8 and 40° C for 4 hours)	(−)	(−)	(+)
(Casein solution 15 ml. +8% boiled Trypsin solution 5 ml.) (same as above)	(+)	(+)	(+)
(Casein solution 15 ml. +8% Pepsin solution 5 ml.) (at pH 1.8 and 37°C for 4 hours)	(−)	(−)	(+)
(Casein solution 15 ml. +8% boiled Pepsin solution 5 ml.) (same as above)	(+)	(+)	(+)

Remark; In this table the sign (−) indicates no colour or sedimentation reaction and (+) positive case.

About the saline solution of the substance obtained in the preceding section the protein concentration was estimated and to the solutions the proteolytic enzymes purified as above were added in the calculated volume from the above results equivalently and digested at pH 8.0 in the case of trypsin and at pH 1.8 in the case of pepsin for 4 hours. By this treatment the sedimentation reactions disappeared entirly, but biuret reaction still remained.

The mixtures after digestion were heated at 56° C for 30 minutes in order to inactivate the enzymes. 6 ml. of these mixtures was injected into rabbits and the influences upon the blood calcium content were observed.

Table VIII

Calcium content of blood influenced by the injection of digested substance with trypsin or pepsin

Digested with	Trypsin				Trypsin inactivated by boiling	
No. of rabbit	13		14		15	
Before the experiment	mg. 18.24 %	Diminution	mg. 15.45 %	Diminution	mg. 16.48 %	Diminution
4 hours after an injection	18.24	0	15.45	0	15.48	−6.3%
7 hours after an injection	17.10	−6.2%	14.42	−6.7%	14.42	−12.5

Digested with	Pepsin				Pepsin inactivated by boiling	
No. of rabbit	16		17		18	
Before the experiment	mg. 19.38 %	Diminution	mg. 18.24 %	Diminution	mg. 20.52 %	Diminution
4 hours after an injection	18.15	−6.9%	17.32	−5.0%	18.68	−9.0%
7 hours after an injection	17.57	−7.8	17.10	−6.2	18.01	−12.2

From the results it can be seen that the activity of the substance to lower the calcium content of blood is obviously removed by the digestion by proteinase such as trypsin or pepsin.

7. Fractionation of the water extract of the parotid gland tissue

In the previous sections it becomes almost clear that the substance of the parotid gland is of protein nature. Then further I examined that when the water extract of the parotid gland is fractionated by dissolving ammonium sulfate in the concentration of half saturation or saturation, in which fraction the active substance proceeds. In the saline solution of the parotid gland the ammonim sulfate is dissolved in the concentration of half saturation and let stand half an hour.

The supernatant liquid obtained is removed by centrifugation and the sediment is washed twice with the half saturated ammonium sulfate solution. The sediment is removed by means of small amount of water into the collodium membran and dialysed against running water until no more sulfate ion can be detected in the inner solution. This is the globulin fraction.

The supernatant liquid and the washing solution are combined and in it ammonium sulfate is dissolved to a saturation. The sediment there occurs is collected and dialysed in the collodium membran in the same way described as above. This fraction is the albumin. The both fractions are diluted to a volume as same to the original saline solution, and in it sodium chloride is dissolved to a concentration of 0.85 per cent. 6 ml. of the each solution is injected into rabbit and the influence upon the calcium content of blood was observed. The results are shown in the Table IX.

TABLE IX

The influence of the injection of albumin and globulin fractions of the water extract of the gland

Material for an injection	Globulin fraction			
No. of rabbit	19		20	
Before an experiment	14.73 mg.%	Diminution	16.25 mg.%	Diminution
4 hours after an injection	13.39	−9.9%	15.08	−7.2%
7 hours after an injection	13.70	−6.7	14.86	−8.5

Material for an injection	Albumin fraction			
No. of rabbit	21		22	
Before an experiment	17.72 mg.%	Diminution	15.43 mg.%	Diminution
4 hours after an injection	15.45	−12.8%	13.23	−14.2%
7 hours after an injection	14.42	−18.6	12.47	−19.5

The results indicate that the substance is contained in the albumin fraction almost entirely.

8. *Influence of heat on the activity of the substance*

In order to know what effect exerts the heat against the activity of the substance, the present experiment was performed. The water extract of the gland perpared described as above was heated at 75°C or 60° C for 30 minutes respectively, and 6 ml. of each preparation is injected into rabbits and examined the change of the calcium content of blood.

The results are as follows.

TABLE X

The effect of heat upon the activity of the substance

Heated at	60° C			
No. of rabbit	23		24	
Before an experiment	17.32 mg.%	Diminution	18.08 mg.%	Diminution
5 hours after an injection	14.82	−16.8%	15.36	−15%

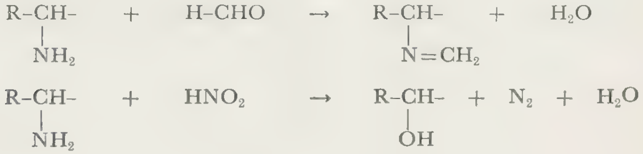
Heated at	75° C				Control	
No. of rabbit	25		26		27	
Before an experiment	mg 18.69 %	Diminution	mg. 16.75 %	Diminution	mg. 19.38 %	Diminution
5 hours after an injection	17.10	−8.4%	15.42	−9%	15.96	−17.6%

The activity of the substance seems to be detroyed by heating at 75° C. for 30 minutes.

10. The relation between the activity of the substance and the ree amino- or carboxyl-groups of the substance

For the purpose to know that whether the free amino- or carboxyl-groups of the substance have any relation for its action or not, I tried to cover the amino-groups by reacting formic aldehyde or sodium nitrite and carboxyl-groups by ethylalcohol.

Desamination; As well known formic aldehyde or sodium nitrite combine with amino groups in the following manners.



$$R\text{-}\underset{NH_2}{\underset{|}{CH}}\text{-} \quad + \quad H\text{-}CHO \quad \rightarrow \quad R\text{-}\underset{N=CH_2}{\underset{|}{CH}}\text{-} \quad + \quad H_2O$$

$$R\text{-}\underset{NH_2}{\underset{|}{CH}}\text{-} \quad + \quad HNO_2 \quad \rightarrow \quad R\text{-}\underset{OH}{\underset{|}{CH}}\text{-} \quad + \quad N_2 \quad + \quad H_2O$$

To 10 ml. of the water extract of the dried gland powder 6 ml. of 30% formol or 30% sodium nitrite solution is added respectively, and after standing for 30 minutes an excess of formol or sodium nitrite are removed by dialysing them in the collodium membran. An epuivalent amount of the substance thus treated was injected into rabbit and observed the change of the calcium content of blood.

TABLE XI

Treated	HCHO			
No. of rabbit	28		29	
Before an experiment	19.38 mg.%	Diminution	17.10 mg.%	Diminution
5 hours after an injection	19.38	0	17.10	0

Treated with	$NaNO_2$				Control	
No. of rabbit	30		31		32	
Before an experiment	18.24 mg.%	Diminution	16.95 mg.%	Diminution	16.24 mg.%	Diminution
5 hours after an injection	18.24	0	16.57	−2.3%	13.31	−18%

Esterfiing; I knew that the active substance is collected in the sediment produced by acidifiing the water extract of the gland at pH 5, 4, so I dried the sediment thus obtained and 30 mg. of it was suspended in 15 ml. of absolute alcohol and dried hydrogenchloride gas was passed through it for ten minutes. The sediment was separated by filtration and suspended again in 15 ml. of adsolute alcohol and the same treatment was repeated as above. The both filtrates are jointed and evaporated at the temperature lower than 40° C to dryness. This substance is suspended in 0.1 ml. of adsolute alcohol and emulsified by adding of physiological saline solution. After 30∼60 minutes after emulsifiing 6 ml. of it is injected into rabbits. The calcium contents of blood influenced by the injection are shown in table 12.

TABLE XII

Material for injection	Esterified				Control	
No. of rabbit	33		34		35	
Before an experiment	19.24 mg. %	Diminution	15.96 mg. %	Diminution	20.52 mg. %	Diminution
5 hours after an injection	17.10	−6.3%	14.82	−6.5%	15.96	−22.2%

As can be seen from the Tables the amino-groups and carboxylradicals of the substance are necessary for its activity.

11. The experiment with the another organs than parotid gland

To determine that the action of lowering the calcium content of blood is specific to the parotid gland, the same experiments were performed using the water extract of another organs such as liver, kidney or spleen. The organs were treated as same as in the case of the parotid gland and the water extract was obtained.

The concentrations of the protein of these extracts are estimated and equivalent amount of these extract as it from the parotid gland was injected into rabbit and as it is illustrated in the next table no influences upon the calcium content of blood could not be observed except in the case of spleen.

TABLE XIII

Liver extract

No. of rabbit	36		37	
Before an injection	17.60 mg.%	Diminution	18.70 mg.%	Diminution
3 hours after an injection	17.16	−2.5%	18.70	0
6 hours after an injection	18.70	−6.2	16.50	−11.8%

Kidney extract

No. of rabbit	38		39	
Before an injection	19.80 mg.%	Diminution	17.60 mg.%	Diminution
3 hours after an injection	17.72	−5.5%	16.72	−5.0%
6 hours after an injection	17.72	−5.5	16.50	−5.6

Spleen extract

No. of rabbit	40		41	
Before an injection	18.70 mg.%	Diminution	19.80 mg.%	Diminution
3 hours after an injection	15.40	−5.5%	16.50	−16.7%
6 hours after an injection	16.50	−5.5	17.60	−11.1

The active substance in the spleen tissue can be also collected in the albumin fraction when the water extract is fractionated by ammonium sulfate. To know the active substance in parotid gland and spleen tissues are identical or not, the serological test was performed. The rabbit was immunized with the albumin fraction of water extract of the parotid gland three times and with the immun serum thus obtained and the albumin fraction from the gland or spleen as an antigen the complement fixation reactions were tested. The results are shown in the next Table. In the table, cases where no hemolysis occur are indicated with (−), and complete hemolysis with (⧻), while for intermediate cases the sign (+) and (⧺) are used.

Table XIV

The complement fixation reaction

Antibody producer; Albumin from ox's gland.

Antibody detector; The same as above.

No. of rabbit.	Unit of complement				Antigen + Complement	Antiserum + Complement
	2	4	6	8		
42	(−)	(−)	(−)	(−)	(卌)	(卌)

Antibody producer; Albumin from ox's gland.

Antibody Detector; Albumin from ox's spleen.

No. of rabbit.	Unit of complement				Antigen + Complement	Antiserum + Complement
	2	4	6	8		
42	(+)	(卌)	(卌)	(卌)	(卌)	(卌)

Thus the active substance from the parotid gland and the spleen are not same at least serologically.

12. *The comparison of the active substances from the ox's and horse's gland*

Further the author continued the study concerning the horse's parotid gland and knew that the horse's gland also contained the substance which lowers the calcium content of blood. The active substance can be collected in the albumin fraction of the water extract of the gland as in the case of an ox.

Table XV

Influence upon calcium content of blood by the injection of the albumin fraction of horse's parotid gland

No. of rabbit	43		44	
Before an injection	18.54 mg.%	Diminution	16.94 mg.%	Diminution
5 hours after an injection	15.45	−16.6%	13.85	−18.2%

The serological identification between both albumins from ox's and horse's parotid gland was tested. The results are as follows.

Table XVI

The complement fixation reaction

Antibody producer; Albumin from ox's gland.
Antibody detector; The same as above.

No. of rabbit.	Unit of complement				Antigen + Complement	Antiserum + Complement
	2	4	6	8		
42	(−)	(−)	(−)	(−)	(⧻)	(⧻)

Antibody producer; Albumin from ox's gland.
Antibody detector; Albumin from horse's gland.

No. of rabbit.	Unit of complement				Antigen + Complement	Antiserum + Complement
	2	4	6	8		
42	(+)	(⧻)	(⧻)	(⧻)	(⧻)	(⧻)

Form these results, the active substance of ox's and horse's gland differs from each other serologically.

SUMMARY

(1) The substance which is contained in the parotid gland and lowers the calcium content of blood is soluble in water, but not in alcohol, ether or acetone.

(2) The active substance indicates various protein natures, but no reduction reaction also after hydrolised it with acid.

(3) The substance loses the activity by digestion with proteinase such as trypsin or pepsin.

(4) The substance does not pass through the semipermeable membran.

(5) The substance is inactivated by heating at 75° C for 30 minutes. From these properties the active substance as the deteriorator of the calcium content might be considered as

protein like substance.

(6) The amino-groups and carboxyl-groups of the substance are necessary for the activity of the substance.

(7) The active substance is collected in the albumin fraction at the fractionation by ammonium sulfate.

(8) The water extract of ox's kidney and liver do not possess the lowering effect of the calcium content of blood, but the extract of spleen indicates the lowering effect slightly. Also in this case the active substance can be collected in the albumin fraction.

(9) The active substance from the gland and spleen differs each other serologically.

(10) The substance lowering the calcium content of blood is contaminated also in the horse's parotid gland especially in its albumin fraction. The substances from the parotid gland of different animals differ each other serologically.

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STUDIES ON THE MECHANISM OF THE TOXIC ACTION OF ORGANIC HALOGEN COMPOUNDS

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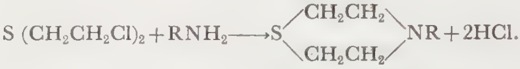
Several explanations had been put forward long before World War II to account for the toxic action of certain noxious gases including organic halides (mustard gas and related compounds, phosgene, chloropicrine, chloroacetophenone, benzyl bromide, ect.), viz. the intracellular acid formation hypothesis, (1) the active halogen radicale hypothesis (2), the amino combination hypothesis (3), the lipid reaction hypothesis (4), etc. None of them, however, proved to maintain their validity and, in particular, the intracellular acid formation hypothesis which assumed that traces of hydrogen halide liberated as the result of intracellular hydrolysis might be correlated with their toxicity on the living cell, had been almost abandoned because it was incompatible with a number of facts. The amine combination hypothesis as the most powerful alternative drew much attention for some time but seemed far from complete approval as yet.

We carried out, during the past war time, some fundamental experiments *in vitro* concerning the mechanism of their toxicity, and after we obtained decidedly negative results in respect to the validity of the amine reaction hypothesis, we were led through extensive investigations to the conclusion that the combination with the sulfhydryl groups of proteins, particularly enzyme proteins would play the most significant rôle in the toxic action of these halides (5).

EXPERIMENTAL

1. Re-investigation of the Amine Reaction Hypothesis.

This hypothesis was brought forward directly after World War I by Lawson, Reid & Allen, (6) on the basis of the experimenta. evidence that under certain conditions, though rather drastic, condensation occurred between organic halides of more or less toxicity and the amino groups of certain amino acids, as pointed out by Helfrich & Reid (6), Cashmore & McComby (7) and others. In case of mustard gas ($\beta\beta'$-dichloroethyl sulfide), for instance, the reaction proceeds as follows:


$$S(CH_2CH_2Cl)_2 + RNH_2 \longrightarrow S\begin{matrix} \diagup CH_2CH_2 \diagdown \\ \diagdown CH_2CH_2 \diagup \end{matrix}NR + 2HCl \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

(4–R–1,4 thiazane)

$$S(CH_2CH_2Cl)_2 + 2RNH_2 \longrightarrow S(CH_2CH_2NHR)_2 + 2HCl \quad \ldots\ldots\ldots\ldots\ldots\ldots (2)$$

(Bis β–R–aminoethyl sulfide)

In view of the observations, they suggested the possibility that the disappearance of the essential amino groups of the proteins might lead to anomalous changes of pathobiosis in living tissues. Muntsch and his associates (8) gave a very positive support to the theory.

We commenced through experimental re-investigation of the former results, the fundamental principles thereof being, firstly, to extend the variety of the halides to a sufficient degree, secondly, to select the serum protein as the most suitable object of combination, thirdly, to carry out the experiments under nearly physiological conditions, and fourthly, to estimate the degree of reaction by the formol titration. At first, we carried out some preliminary experiments using glycine as the representative of amino acids, but the experimental details will not be discussed here.

Methods

To a mixture of 30 ml. of horse serum and 30 ml. of bicarbonate-carbonate buffer usually 0.5 g of an organic halide was added and the reaction proceeded at nearly 30°C or so with frequent shaking and addition of 2*N*. NaOH, the alkalinity of the medium being maintained in the range of pH 8.4 ± 0.5. It may be added that the experimental technique of Berenblum & Wormall (9) in the preparation of the mustard gas- or sulfone-treated protens was applied. After the completion of the reaction, the eventual decrease of the free amino groups of the protein was determined on each 40% of the mixture with $N/5$ NaOH by the formol titration, modified by Hirade (10), the borax solution of pH 9.2 to which formol was added in the same amount as in the titration mixture, being used as the most suitable standard.

And in this connection, it must be added that a new observation was made that, under exact constancy of pH, the addition of formol inhibits the color of phenolphthaleine to a very marked degree (10). Similar phenomena were seen in cases of other dyes, especially, thymol blue. This so-called the "formol error of the pH indicators" might be attributed to the reversible formation of a less colored complex between formol and dye ion.

Results

The most important part of the experimental results concerning the reactivity of twenty-five organic halides (including chemical warfare gases and their derivatives) on the amino groups of the horse serum protein, is summarized in Table I, where "n" is the number of the active halogen groups in each halide molecule, the amount of the bound amino ("$\varDelta NH_2$") and its percentage to the total halide ("$\Delta NH_2\%$") are calculated from the results of formol titration and the percentage of hydrolysis ("Hyl%") shows the residual, uncombined amount. Further, the reactivity "R" is a qualitative measure for the intensity of combination and the toxicity "Tox" of each halide is given as a qualitative representation of the degree of toxicity, calculated chiefly on the basis of the data described by Handerson & Haggard (11) and the values of lethality products (=conc.×time) proposed by Muntsch. (12)

As seen in the foregoing Table, one might have the general impression that the reactivity, except a few cases, is very slow and feeble, in spite of the relatively higher concentrations of both reactants. Let

TABLE I. *Reaction of Organic Halides with Serum Protein*

Ser-prot-amino 0.792 m*M* tot. vol. 24 ml., pH 8.4, 28 ± 2°C.

Compound	Formula	Mol. Wt.	Amount g	Amount m*M*	n	ΔNH_2 m*M*	,, %	Pyl %	R	Tox
ββ′—Dichloroethyl sulfide	$S(CH_2CH_2Cl)_2$	159	0.20	1.26	2	0.132	5.2	94.8	+	+++
ββ′—Dichloroethyl sulfoxide	$SO(CH_2CH_2Cl)_2$	175	0.20	1.14	2	0.032	1.4	0 ?	±	±
ββ′—Dichloroethyl sulfone	$SO_2(CH_2CH_2Cl)_2$	191	0.20	1.05	2	0.568	27.1	72.9	+++	+++
αα′—Dichloroethyl sulfide	$S(CHClCH_3)_2$	159	0.20	1.26	2	0.224	9.0	91.0	++	±
αα′ ββ′—Tetrachloroethyl sulfide	$S(CHClCH_2Cl)_2$	228	0.20	0.877	4	0.346	9.9	90.1	++	+
Phosgene	$COCl_2$	99	0.23	2.33	2	0.374	7.4	92.6	++	++++
Diphosgene	$ClCOOCCl_3$	198	0.074	0.373	4	0.177	11.9	88.1	++	++++
Chloropicrine	CCl_3NO_2	164.5	0.20	1.22	3	0.020	0.55	0	−	++
Bromoacetone	CH_3COCH_2Br	136.5	0.20	1.47	1	0.076	5.2	94.8	+	++
Monoiodoacetate	ICH_2COOH	186	0.20	1.08	1	0.032	3.0	0 ?	±	+++
Monochloroacetate	$ClCH_2COOH$	94.5	0.20	2.12	1	0	0	0	−	±
Chloroacetophenone	$C_6H_5 \cdot COCH_2Cl$	154.5	0.20	1.30	1	0.026	2.0	0 ?	±	++
Benzyl bromide	$C_6H_5 \cdot CH_2Br$	171	0.20	1.17	1	0.132	11.3	?	++	++
Benzyl chloride	$C_6H_5 \cdot CH_2Cl$	126.5	0.20	1.58	1	0.058	3.7	0 ?	±	±
p-Nitro benzyl chloride	$NO_2 \cdot C_6H_4 \cdot CH_2Cl$	171.5	0.20	1.17	1	0	0	0	−	+
Dimethyl sulfate	$(CH_3)_2SO_4$	126	0.20	1.59	1	0.190	12.0	88.0	++	+++
Diethyl sulfate	$(CH_2H_5)_2SO_4$	154	0.20	1.30	1	0.056	4.3	95.7	+	(+)
Butyl chloride	$CH_3CH_2CH_2CH_2Cl$	92.5	0.20	2.16	1	0.006	0.3	0	−	−
Allyl chloride	CH_2CHCH_2Cl	76.5	0.20	2.61	1	0.036	1.4	0	±	+
Ethylene bromide	$(CH_2Br)_2$	188	0.20	1.06	2	0	0	0	−	−
Ethylene chlorohydrin	$CH_2Cl \cdot CH_2OH$	80.5	0.20	2.48	1	0.006	0.25	0	−	−
Chloroform	$CHCl_3$	119	0.20	1.68	3	0	0	0	−	−
Benzoyl chloride	$C_6H_5 \cdot COCl$	140.5	0.20	1.42	1	0.432	30.3	69.7	+++	(+)
Acetyl chloride	CH_3COCl	78.5	0.20	2.55	1	0.012	0.5	99.5	−	(±)
Benzene sulfochloride	$C_6H_5 \cdot SO_2Cl$	176.5	0.20	1.13	1	0.442	39.0	61.0	+++	(+)

us consider, first of all, the correlation between the reactivity and the toxicity of the limited group of mustard gas and its derivatives. Eventhough the relative behavior of the practically harmless sulfoxide and the highly toxic sulfone be compatible with the theory, the only feeble reaction of mustard gas compared to that of $\alpha\alpha'$-dichloroisomer (or $\alpha\alpha'$ $\beta\beta'$-tetrachloro sulfide) of far less toxicity remains unexplained. Moreover, the experimental fact that mustard gas itself does not combine with the serum protein at pH 7.4, would render the amine reaction hypothesis that had been pro-posed in order to explain the mechanism of its own toxicity almost invalid.

But it would not be pertinent to say that the results obtained by Berenblum & Wormall (9) that the sulfone or mustard gas treatment of horse serum initiates an immunological reaction of new specificity, might be contradictory to the observations mentioned above, because their experiments were carried out at about pH 8.0. Moreover, the fact that the immunological reaction of sulfone was decidedly stronger than that of mustard gas, coincides with our results concerning the relative intensity of amino combination of both.

Among other toxic gases, phosgene and diphosgene, both the highest toxic, reacted more or less but only relatively weakly. Chloropicrine, monoiodoacetate and chloroacetophenone reacted only trace and it was only dimethyl sulfate, though itself not a halide, that reacted with sufficient intensity comparable to its toxicity. Benzoyl chloride and benzene, sulfochloride, both far less toxic, reacted the most strongly of all.

To examine, as a whole, the correlation between the reactivity and the toxicity of all compounds, following statistical procedure was taken. If we divide the whole, from the view-point of reactivity, into the stronger (≧ ++) and the weaker (≦ ++) reacting groups and, from the view-point of toxicity, into the higher (≧ ++) and the lower toxic (≦ ±) categories, a correlation table as Table II will be obtained, X^2

TABLE II. *Correlation between Amine Reaction and Toxicity*

Reaction \ Toxicity	Higher		Lower		Tot.
Stronger	Sulfone Diphosgene Dimethyl sulfate	$n_1=3$	$\alpha\alpha'$-Dichloroethyl sulfide Tetrachloroethyl sulfide Benzoyl chloride Benzene sulfochloride	$n'_1=4$	7
Weaker	Phosgene Mustard gas Chloropicrine Bromoacetone Monoidoacetate Chloroacetophenone Benzyl bromide	$n_2=7$	Sulfoxide Benzyl chloride p. NO_2-benzyl chloride Diethyl sulfate Allyl chloride Ethylene chlorohydrin Acetyl chloride Monochloroacetate Butyl chloride Ethylene bromide Chloroform	$n'_2=11$	18
Tot.	10		15		25

$X_2=0.075$

being calculated equal to 0.075, so that not any significant correlation could be acknowledged between the reactivity and the toxicity of the whole system. In other words, we believe to have arrived at an entirely negative conclusion concerning the validity of the amine reaction hypothesis.

II. Experimental Studies on the Reaction between Organic Halides and Reduced Glutathione

Some time before, Ninomiya had made a new discovery that the amount of the reduced glutathione (GSH) of the whole blood is decreased by the action of mustard gas with a considerably high rate, as shown in the following equation:

$$S(CH_2CH_2Cl)_2 + 2GSH \rightarrow S(CH_2CH_2SG)_2 + 2HCl \text{ ....} (3)$$

Such a type of reaction, we supposed, might possibly play some rôle in the mechanism of toxicity of these compounds, so that we were led to the commencement of some fundamental investigations *in vitro* concerning the thiol reaction of the whole halides toward the reduced glutathione, in analogy to the amine combination experiments previously described.

Methods

The halides tested were almost the same as in the foregoing experiments. To each of three or more mixtures containing 6 ml. of fresh aequeous solution of 0.2% GSH and 6ml. of *M*/5 bicarbonate the same small amount of halide sample (2—10 mg. or more) was added, dissolved by shaking and the reaction was followed at exactly 30°C, the alkalinity of the medium being maintained constant within the range of pH 8.0 ± 0.5 by occasional addition of a few drops of *N*/5 NaOH. The sulfhydryl combination was determined, from time to time, after slight acidification iodometrically with *N*/100 KIO_3, in view of the technique suggested by Woodward & Fry (13). In most cases, the experiment was continued for one hour or less, partly because the rate of reaction was usually considerably high and partly because the autoxidation of GSH might exert a more or less complicating influence on the exactness of the kinetics.

Results

As in the case of the amino combination already mentioned, the whole view of the reaction of twenty-four organic halides with GSH, is summarized in Table III, where the half reaction time or "50% RT" was added in order to facilitate an exact quantitative study; "ΔGSH" denotes the amount of the combined SH.

In the first place, let us examine the sulfhydryl reactivity of mustard gas and its derivatives in relation to their toxicity. A satisfactory parallelisum of such degree, as has been never experienced in case of amino combination or other reactions, is observed between the reactivity and the toxicity, in as much as mustard gas itself made a rapid reaction rather exceeding sulfone and $\alpha\alpha'$-isomer of very feeble toxicity failed to make any noticeable combination. Moreover, it should be pointed out that mustard gas, in contrast to the case with serum protein, reacted even at pH 7.4.

Further important preponderance of the sulfhydryl reaction over the amino combination is the fact that chloropicrine, bromoacetone, monoiodoacetate, chloroacetophenone and benzyl bromide, which combined only feebly or trace with serum protein, now reacted with GSH with sufficient intensity just comparable to their toxicity. In general a fair parallelism is apparent between the thiol reactivity and the toxicity of the halides, with exception of less toxic benzoyl chloride, benzene sulfochloride and benzyl chloride which reacted with exceeding intensity. To elucidate what degree of correlation exists between the thiol reactivity and toxicity of the whole compounds, the same method was adopted as in the case of the amino combination. If we divide them, from the view-point of reactivity, into the stronger (≧ ⧺) and the weaker (≦ +) reacting groups and further, from the view-point of toxicity, into the higher (≧ ⧺) and the lower (≦ +) toxic categories, the following Table (IV) will be obtained,

TABLE III. *Reaction of Organic Halides with Reduced Glutathione*

GSH 0.0395 mM tot. vol. 12cc, pH 8.0, 30°C.

Compound	Formula	MW	Amount g	Amount mM	n	50% RT	ΔGSH mM	,, %	Hyl %	R	Tox
ββ′-Dichloroethyl sulfide	$S(CH_2CH_2Cl)_2$	159	2.6	0.016	2	3.5′	0.023	72	28	+++	+++
ββ′-Dichloroethyl sulfoxide	$SO(CH_2CH_2Cl)_2$	175	10.0	0.057	2	∞	0	0	0	−	±
ββ′-Dichloroethyl sulfone	$SO_2(CH_2CH_2Cl)_2$	191	3.0	0.016	2	15′	0.030	94	6	+++	+++
αα′-Dichloroethyl sulfide	$S(CHClCH_3)_2$	159	6.0	0.038	2	—	0?	0?	100?	±	±
αα′ββ′-Tetrachloroethyl sulfide	$S(CHClCH_2Cl)_2$	228	3.8	0.016	4	>60′ compl.	0.020	31	69	++	+
Phosgene	$COCl_2$	99	2.7	0.027	2	<5′ compl.	0.032	60	40	+++	++++
Diphosgene	$ClCOOCCl_3$	198	2.4	0.012	4	,,	0.027	58	42	+++	++++
Chloropicrine	CCl_3NO_2	164.5	3.4	0.021	3	,,	>0.008	100	0	++++	++
Bromoacetone	CH_3COCH_2Br	136.5	3.0	0.022	1	<2′	0.024	110	0	++++	++
Monoiodoacetate	ICH_2COOH	186	3.5	0.019	1	4′	0.021	110	0	+++	+++
Monochloroacetate	$ClCH_2COOH$	94.5	25.0	0.26	1	6 hrs.?	0.006	2	0	±	±
Chloroacetophenone	$C_6H_5 \cdot COCH_2Cl$	154.5	2.5	0.016	1	2′	0.017	105	0	++++	++
Benzyl bromide	$C_6H_5 \cdot CH_2Br$	171	3.6	0.021	1	2.5′	0.022	105	0	++++	++
Benzyl chloride	$C_6H_5 \cdot CH_2Cl$	126.5	6.0	0.048	1	9′	×0.023	>48	0	++	±
Dimethyl sulfate	$(CH_3)_2SO_4$	126	3.8	0.030	1	15′	0.022	73	27	+++	+++
Diethyl sulfate	$(C_2H_5)_2SO_4$	154	12.2	0.080	1	18′	0.011	14	86	+	(+)
Butyl chloride	$CH_3CH_2CH_2CH_2Cl$	92.5	22	0.24	1	∞	0	0	0	−	−
Ethylene bromide	$(CH_2Br)_2$	188	20.5	0.11	2	—	0.003	3	0	±	−
Ethylene chlorohydrin	$CH_2Cl \cdot CH_2OH$	80.5	19.1	0.24	1	—	0.001	0.4	0	−	−
Chloroform	$CHCl_3$	119	30	0.25	3	∞	0	0	0	−	−
Carbon tetrachloride	CCl_4	154	32	0.21	4	∞	0	0	0	−	−
Benzoyl chloride	$C_6H_5 \cdot COCl$	140.5	4.1	0.030	1	5′ compl.	0.020	67	33	+++	(+)
Acetyl chloride	CH_3COCl	78.5	16.8	0.21	1	—	0	0	100	−	(±)
Benzene sulfochloride	$C_6H_5 \cdot SO_2Cl$	176.5	4.2	0.024	1	∖10′ compl.	>0.034	>140	0	++++	(+)

TABLE IV. *Correlation between Sulfhydryl Reaction and Toxicity*

Reaction \ Toxicity	Higher	Lower	Tot.
Stronger	Mustard gas Phosgene Chloropicrine Monoiodoacetate Benzyl bromide Sulfone Diphosgene Bromoacetone Chloroacetophenone Dimethyl sulfate $n_1=10$	Benzyl chloride Benzoyl chloride Benzene sulfochloride $n'_1=3$	13
Weaker	— $n_2=0$	Sulfoxide Tetrachloroethyl sulfide Diethyl sulfate Butyl chloride Ethylene chlorohydrin Carbon tetrachloride $\alpha\alpha'$-isomer Monochloroacetate Ethylene bromide Chloroform Acetyl chloride $n'_2=11$	11
Tot.	10	14	24

$P=\frac{1}{6860}$

whereof it is noteworthy that all stronger (weaker) toxic halides reacted unexceptionally stronger (weaker).

We can deduce, therefore, a highly significant correlation between the sulfhydryl reaction and the toxicity with a probable error of less than about 0.015% and such a remarkable result is in striking contrast to that of the amino combination and other reactions.

Another fundamental difference is, it might be added, the relative velocities of the amino and thiol combination. Because of the fact that the actual concentrations of the reactants were, in most cases, decidedly higher in case of the amino combination than the sulfhydryl reaction, the true velocity constants of the latter would be uncomparably larger than that of the former, in accordance with the experimental evidence pointed out by Michaelis & Schubert (14). This may lead to an important suggestion that certain toxic compounds, once invaded into the living tissues, would react far more rapidly with thiol than amino groups.

Demonstration of the Combination of the Typical Halides with Sulfhydryl Groups of Tissues

In view of the thiol reaction theory mentioned above, it is presumed that certain toxic compounds would combine with the essential sulfhydryl groups present in the tissue proteins, particularly enzymes, thus causing anomalous phenomena due to deprivation of SH. We carried out, therefore, some experiments *in vitro* with two representative halides (mustard gas and chloropicrine) in evidence of such combination. After some negative results with denaturated egg-white by Rapkine's (15) method and coagulated skin slices according to Walker, (16) we tested the thermostable muscle residue (16) and obtained positive results.

Methods

The thermostable muscle residue was prepared by the method described by Hopkins & Dixon (17) from the skeletal muscle of a rabbit through heat denaturation, the nitroprusside reaction being of intermediary strong degree.

The sulfhydryl groups present in the denaturated tissue was estimated qualitatively by nitroprusside reaction but the decrease of the amount of SH was determined quantitatively by applying the 2, 6-dichlorophenol indophenol method of Todrick & Walker (18). After the completion of the interaction between known amounts of halide and tissue under nearly physiological conditions, the residual mixture was devided homogenously into several portions, to which different volumes of 0.1% indophenol solution were added and sufficient time (fifteen minutes) was given at 100°C. The amount of sulfhydryl groups was calculated from the limit of decoloration according to the formula:

1 mg of indophenol dye=0.351 mg of cysteine.

Results

Preliminary experiments revealed the fact that about 50% reduction in the intensity of the nitroprusside reaction was brought about by nearly 90 mg. of mustard gas for 1 g. of the muscle residue, whereas in case of chloropicrine the inhibition was so strong that 10 mg. sufficed for the almost complete disappearance of the color reaction.

The results of the colorimetric determination of the decrease of thiol in tissues are summarized in Table V. As would have been predicted from the qualitative results mentioned above and also from the relative reactivities toward reduced glutathione already stated, 88 mg. of mustard gas reduced the amount of SH to about one half, whereas only 3.8 mg. of chloropicrine reduced the same to about one fourth of the original.

TABLE V. *Reaction with the Sulfhydryl Groups of Muscle Residue*

Halide	Amount (mg.)	Dye consumed by 1 g tissue (mg.)	SH content of 1 g tissue (as mg. cysteine)
—	0	4.3±0.2	1.51±0.07
Mustard gas	88.0	2.0±0.1	0.70±0.04
Chloropicrine	3.8	1.0±0.2	0.35±0.07

Effect on the Activity of Succinic Dehydrogenase

In connection with the foregoing observations, some experiments were carried out in order to elucidate what influence the action of both toxic halides might have on the activity of succinic dehydrogenase, selected as the most easily handled, though not sensitive enough, representative of the sulfhydryl enzymes.

A crude enzyme material of the ordinary activity was obtained easily from minced thigh muscles of a rabbit. One half g. of this preparation was treated with suitable doses of the halide under physiological conditions with sufficient duration, washed once, centrifuged and the residue was used for the reduction experiment. The methylene blue technique was applied, the details following the method described by Hopkins & Morgan (19). It might be added that the Thunberg tubes were bifurcated, as had been modified by Hirade (20).

Results

The typical results of methylene blue reduction are shown in Table VI. As seen in the table, the incubation of the dehydrogenase with mustard gas or chloropicrine, decreases more or less the enzyme activity, but the former acts far less profoundly than the latter, in accordance with the data obtained in the combination experiments with the thiol groups of tissues.

TABLE VI. *Effect on the Activity of Succinic Dehydrogenase*

Halide	Amount (mg.)	pH of Incubation	Reduction Time
—	0	—	11′
Mustard gas	66.0	7.7	22′
Chloropicrine	3.8	7.3	>3 hrs.

Discussion

We have so far described the outline of our isolated experiments during the war time, the result of which makes it probable that the combination with the sulfhydryl groups of the tissue proteins, particularly enzymes, would play a significant rôle in the toxicity of certain toxic halides. Enzymic studies will be continued further.

Bacq & Goffart (21) had pointed out the similarity of action of several vesicant compounds (including mustard gas and chloropicrine) to that of monoiodoacetic acid, by studying their effects on the muscle contraction. Supported by the further evidence (22) that chloropicrine reacts with the sulfhydryl groups of egg albumin, myosin and papain, they were, a few years ago, led to the proposal of a new class name of drugs "thioloprives" (23) or thiol antagonists which includes all substances reacting or interfering with the thiol functions of proteins, cysteine or glutathione. Oxidizing agents, heavy metals, maleic acid and halogenated organic substances like mustard gas, lewisite or iodoacetic acid are included. The creation of this new pharmacological family is justified by the fact that its members have many common toxic effects (Lundsgaard contracture, cytotoxic action, edema) and inhibit the same enzymes. Also, a logical interpretation of many of their actions is possible on the basis of their reactions with thiol groups.

In connection with the view mentioned above, it is of fundamental importance that Barron & Singer (24) demonstrated the presence of sulfhydryl groups was essential for the activity of a large number of enzymes concerned with the metabolism of carbohydrate, protein and fat.

Berenblum & Wormall (9), studying the immunological properties of the mustard gas- and sulfone-treated serum proteins as already quoted, arrived at the natural assumption that the reaction discussed might be concerned with the free amino groups of the protein, thus giving support to the amine reaction hypothesis. But during World War II, their view seemed, just as in the case of our isolated studies already stated, to turn from the amine to the thiol reaction, in so far as they were faced with the observation that mustard gas does not react with almost any of the amino acids or the amino groups of proteins, whereas it does react readily with the sulfhydryl of cysteine. (25) It was also noted at least qualitatively that as a result of the reaction of mustard gas, the free and titratable SH of the various proteins are greatly decreased.

Conclusion

Eventhough one could hardly ascribe all the observations stated above to one and sole thiol reaction, it would be pertinent to say that the most important mechanism of the toxic action of certain organic halides will be related to their combination with essential sulfhydryl groups of tissues, especially enzyme proteins.

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THE ANTIGENIC ACTIVITY OF CHOLESTEROL*

By

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Using the technic of complement fixation, Sachs and Klopstock (1925) (1) first reported that cholesterol showed the antigenic activity when rabbits were injected with it mixed with swine serum. Their results were confirmed by Ornstein (1926) (2), Plaut and Kassowitz (1930) (3). Ito (1928, 1929) (4) and Sudo (1930) (5) also reported that the mixtures of cholesterol and swine serum produced the specific antisera which reacted only with cholesterol and not with lecithin or other lipids. Weil and Besser (1931, 1932) (6) investigated also the specificity of such antisera, using dihydrocholesterol, cholesterol oxide, oxycholesterol, cholesterol acetate, cholesterol palmitate or cholesterol dibromide as a test antigen. Further, Weil and den Dooren de Jong (1937) (7) obtained the similar results by means of the specific flocculation. Berger and Scholer (1932) (8) investigated the antigenic action of cholesterol and ergosterol, and the specificity of the antisera produced by them. Aoki (1938) (9) reported the injections of the mixtures of cholesterol and "adsorbin" (aluminium silicate) also produced the antibody, which showed complement fixation and flocculation with cholesterol. Furthermore, Hirose (1934) (10) obtained the active anti cholesterol serum by the percutaneous immunization with lanolin.

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As for other lipids, lecithin was also reported to have antigenic properties by many investigators (Sachs and Klopstock (1925) (1), Ornstein (1926), (2) Ito (1929), (4) Sudo (1930) (5), Plaut and Rudy (1932) (11), but Levene, Landsteiner and van der Sheer (1927) (12), Kimizuka (1935) (13) and Fujimura (1937) (14) reported that the purified lecithin had no antigenic activity. Is there any possibility that cholesterol also loses its antigenic activity when it is purified completely? Onishi (1937) (15) stated it questionable that such a normal constituent of animal tissues as cholesterol has the antigenic activity. Wadsworth, Maltaner and Maltaner (1935) (16) investigated this problem and concluded that the reactions of such a serum are non-specific and due to its increased anticomplementary property, together with fluctuations in the stability of cholesterol suspensions caused by the changes in the amounts of protective serum colloids. But Hahn and Hazato (1936) (17) disagreed with this opinion. They compared the non-specific anticomplementary property with the specific complement fixation under various conditions and concluded that the effect of the fluctuations in the stability of cholesterol suspensions could not be the sufficient ground for denying the specific serological reactions of this immune serum.

In the experiments to be reported in the present paper attempts were made to differentiate the anticomplementary property and fluctuations of colloidal stability of cholesterol from the specific serological reactions with the sera from the rabbits inoculated with the mixtures of purified cholesterol and swine serum, and to clarify the antigenic activity of cholesterol, as measured by complement fixation and flocculation tests.

EXPERIMENTAL

Methods

Preparation of Colloidal Solution of Cholesterol—Commertially prepared crystalline cholesterol (Takeda) was recrystallized three times from absolute alcohol. The melting point of this crystal was 147.5-148°C.

5 ml. of 1.0 per cent solution of this recrystallized cholesterol in absolute alcohol were poured drop by drop into 50 ml. of boiling water, stirring good with a glass rod, and filtered hot. This 0.1 per cent colloidal suspension of cholesterol in water was used for the antigen in complement fixation and flocculation tests.

20 ml. of one per cent cholesterol solution in absolute alcohol were poured drop by brop into 100 ml. of boiling water, stirring good, filtered immediately, and, after cooling, 5 ml. of fresh obtained swine serum were mixed with the filtrate. 9 ml. of 10 per cent sodium chloride solution and some water were added to the mixture to make the final volume up to 108 ml. This procedure must be performed at the temperature below 20°C. After one hour's standing in an ice box, this solution was used for the immunization of rabbits.

The Preparation of Cholesterol dibromide—5 gm. of recrystallized cholesterol were dissolved in 50 ml. of ether, and mixed under ice-cooling with 1.6 ml. of bromine dissolved in 5 ml. of ether. After 30 minutes this cold solution was added to 25 ml. of cold glacial acetic acid and the separated crystalline bromide was washed repeatedly with a mixture of ether and glacial acetic acid 2 : 1, then with glacial acetic acid, and finally with alcohol. After dried completely, it was recrystallized from alcohol. Melting point 111°C.

The Preparation of Cholesterol from Cholesterol dibromide—To the alcoholic solution of cholesterol dibromide the half quantity of sodium iodide was added. The solution was boiled for an hour and, after cooling, it was poured into a large volume of cold sodium sulfite solution, and the mixture was stirred good. Crystalline cholesterol was separated, and it was recrystallized several times from alcohol.

Preparation of Cholesterol acetate—The mixture of 5 gm. of dried cholesterol and 10 gm. of glacial acetic acid was boiled for an hour, and the solution was poured into a large volume of cold water. The acetate crystals were filtered, washed with water and recrystallized repeatedly from ether and from hot alcohol. Melting point 114–115°C.

Saponification of Cholesterol acetate—To the alcoholic solution of cholesterol acetate excess of saturated potassium hydroxide solution was added, and the mixture was boiled on a water bath for an hour and filtered. The alcohol was evaporated from the filtrate on a water bath, and some water was added to the residue. After standing in an ice-box for 24 hours, it was filtered, washed with dilute alcohol, dried, dissolved in a small quantity of ether, and ether was evaporated.

Immunization of Rabbits—Adult rabbits weighing 2500–3000 gm. were inoculated intravenously with 10 ml. of the mixture of cholesterol and swine serum twice a week and a week after eleven inoculations the blood was obtained by heart puncture and the serum was separated from it.

Complement Fixation Tests—Seven units of inactivated antiox hemolysin were incubated at 37°C. with ox erythrocytes for 15 minutes. Complement titers of pooled guinea pig sera were estimated by these sensitized erythrocytes, incubating their mixtures for an hour at 37°C. Complement fixation tests were carried out by progressively increased units of complement, using 10 fold dilutions of antisera, or by progressively doubled dilutions of antisera, using two units of complement.

Flocculation Tests—To the successive dilutions of immune sera cholesterol suspensions were added and after 24 hours at room temperature (higher than 10°C.) the appearance of turbidity or precipitation was observed.

Results

1. Anticomplementary Action of Cholesterol—To each of the successive dilutions of cholesterol two units of complement were added and after incubated for an hour at various temperatures from 5° to 37°C., one unit of sensitized erythrocytes was added. The anticomplementary action of cholesterol was observed when the temperature of incubation was 20°C. or higher than 20°C., but it disappeared completely when the temperature of incubation was 15°C. or lower than 15°C. (Table I). From these results it is concluded that the specific complement fixation can be titrated if the incubation is carried out at 15°C. for an hour.

Table I

Anticomplementary Action of Cholesterol

Temperature of incubation	Dilution of 0.1% cholesterol									No cholesterol
	2	4	8	16	32	64	128	256	512	
37°C.	1	1	1	1	1	2	3	3	4	4
30°	1	1	1	1	2	2	3	4	4	4
25°	1	1	1	2	3	4	4	4	4	4
20°	1	1	2	3	3	4	4	4	4	4
15°	4	4	4	4	4	4	4	4	4	4
10°	4	4	4	4	4	4	4	4		4
5°	4	4	4	4	4	4	4	4		4

4: complete hemolysis, 3: nearly complete hemolysis,
2: incomplete hemolysis, 1: slight hemolysis

2. *Anticomplementary property of Antisera*—The sera obtained from the same rabbits before and after the immunization with the mixture of cholesterol and swine serum were diluted successively, and two units of complement were added. After the incubation for one hour at 37°C. or 15°C. the sensitized erythrocytes were added, and after standing at 37°C. for one hour the hemolysis was read. The anticomplementary property of the immune sera was observed to be increased a little sometimes, but it was never observed with the dilutions lower than three times (Table II). Furthermore, there were no differences between the incubation temperatures of 15°C. and 37°C., and this fact shows that the anticomplementary property of the sera may not possibly result from their cholesterol contents.

TABLE II

Anticomplementary Action of Normal and Immune Sera

Incubation temp.		37°C.						15°C.					
Serum dilution		1	2	3	4	5	No serum	1	2	3	4	5	No serum
No. 184	B							1	2	3	3	4	4
	A							1	2	3	3	4	4
No. 185	B	4	4	4	4	4	4	4	4	4	4	4	4
	A	3	4	4	4	4	4	3	4	4	4	4	4
No. 186	B	3	3	4	4	4	4	3	3	4	4	4	4
	A	2	2	3	4	4	4	2	2	3	4	4	4
No. 187	B	2	3	4	4	4	4	2	4	4	4	4	4
	A	2	3	4	4	4	4	2	3	4	4	4	4
No. 188	B	4	4	4	4	4	4	1	2	3	4	4	4
	A	1	2	3	4	4	4	1	3	3	4	4	4
No. 189	B	3	4	4	4	4	4	3	4	4	4	4	4
	A	2	4	4	4	4	4	2	3	4	4	4	4
No. 190	B	2	3	4	4	4	4	2	4	4	4	4	4
	A	3	4	4	4	4	4	2	4	4	4	4	4
No. 192	B							3	4	4	4	4	4
	A							2	4	4	4	4	4
No. 193	B	3	4	4	4	4	4	4	4	4	4	4	4
	A	4	4	4	4	4	4	3		4	4	4	4

Complement: 2 units; B: before immunization,
A: after immunization.
4: complete hemolysis, 3: nearly complete hemolysis,
2: incomplete hemolysis, 1: slight hemolysis.

3. Complement Fixation Tests of Antisera with Cholesterol—The antisera of the rabbits (No. 184–193) inoculated with the mixture of recrystallized cholesterol and swine serum were tested, and also the sera of those inoculated with cholesterol only (No. 194–197) or swine serum only (No. 165, 166, 172) were examined as control. The sera and complement were incubated at 15°C. for an hour. When the sera were diluted to tenfold, the antisera (No. 184–193) fixed 2∼6

TABLE III

Complement Fixation with Cholesterol (I)

Serum 1 : 10 (ml.)	0.25	0.25	0.25	0.25	0.25	0.5	—
Complement Units	2	4	6	8	10	2	2
0.1% Cholesterol 1 : 5 (ml.)	0.125	0.125	0.125	0.125	0.125	—	0.25
1.7% NaCi (ml.)	0.125	0.125	0.125	0.125	0.125	—	0.25
Incubation	15°C., one hour						
Sensitized erythrocytes (ml.)	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Incubation	37°C., one hour						

Injections made with	No. of Sera	Complement fixation						
Cholesterol and Swine serum	184 B	0	0	0			0	0
	184 A	4	3	2	1	0		
	185 B	0	0	0				
	185 A	4	3	2	1	0		
	186 B	0	0	0				
	186 A	4	3	2	1	0		
	187 B	0	0	0				
	187 A	4	3	2	1	0		
	188 B	0	0	0				
	188 A	4	4	3	2	1		
	189 B	0	0	0				
	189 A	4	4	4	2	1		
	190 B	0	0	0				
	190 A	4	4	2	1	0		
	192 B	0	0	0				
	192 A	4	4	4	3	1		
	193 B	0	0	0				
	193 A	3	3	2	2	1		
Cholesterol	195 B	0	0	0			0	0
	195 A	1	0	0	0	0		
	194 B	0	0	0				
	196–7 A	0	0	0	0	0		
Swine serum	165–6 B	0	0	0			0	0
	172 A	0	0	0	0	0		

B: before immunization, A: after immunization, 4: complete fixation, 3: almost complete fixation, 2: half fixation, 1: slight fixation, 0: no fixation.

units of complement completely, whereas the control sera didn't fix any complement (Table III). The anticomplementary action of the sera cannot affect the results in such dilutions. If the complement fixation was examined by the successive dilutions of the antisera, even dilutions of 40～160 times were able to fix two units of complement, thus the degree of fixation was of far higher degree than the anticomplementary action (Table IV). Also, as the tables indicate, the amboceptor titer was not parallel to the elevation of anticomplementary activity.

Table IV

Complement Fixation with Cholesterol (II)

Serum dilution (0.25 ml.)	10	20	40	80	160	320	640	5	10	no serum
Complement Units	2	2	2	2	2	2	2	2	2	2
0.1% Cholesterol 1 : 5 (ml.)	0.125							—	—	0.25
1.7% NaCl (ml.)	0.125							—	—	0.25
Incubation	15°C., one hour									
Sensitized erythrocytes (ml.)	0.25							0.25		
Incubation	37°C., one hour									
Antiserum no. 189	4	4	4	4	4	3	2	1	0	0
190	4	4	4	3	2	1	0	0	0	0
192	4	4	4	4	3	1	0	0	0	0

4: complete fixation, 3: almost complete fixation,
2: half fixation, 1: slight fixation, 0: no fixation.

4. Flocculation of Cholesterol resulted from Dilution with Saline—before we proceed to the flocculation test, the colloidal instability of cholesterol in saline solutions must be checked. According to the ordinary test method, the immune sera are diluted with saline solutions and the cholesterol suspensions diluted with water are added to them. The concentration of serum protein becomes lower as the dilution proceeds, and thus the precipitation of cholesterol occurs in the dilute range of the sera. This precipitation is protected if some of gelatin solution is added. The activity of gelatin solution as a protective colloid was tested in various concentrations of cholesterol

(Table V). In the conditions of the experiment (see the note of Table V), gelatin protects the precipitation completely, if added to the cholesterol suspensions in the concentration of one-eighth of cholesterol.

Table V

Protective Colloid Activity of Gelatin for Cholesterol Suspension

To 0.25 ml. of various concentrations of gelatin solution 0.25 ml. of various concentrations of cholesterol suspension, 0.25 ml. of water and 0.25 ml. of 0.85% saline were added and the mixture was allowed to stand for 24 hours at room temperature (10°C). The precipitation of cholesterol was indicated by 4, 3, 2, 1 and 0 according to its degree.

Concentration of cholesterol suspension (%)	Concentration of gelatin solution (%)								
	1/10	1/20	1/40	1/80	1/160	1/320	1/640	1/1,280	1/2,560
1/5	0	0	0	1	3	4	4	4	4
1/10	0	0	0	0	1	4	4	4	4
1/20	0	0	0	0	0	1	3	4	4
1/40	0	0	0	0	0	0	1	4	4

5. *Flocculation Test using Gelatin as a Stabilizer*—When the flocculation tests of the immune sera were carried out as usual, the precipitation occurred in the range of lower concentrations of the sera, and this was protected by adding gelatin as a stabilizer in the concentration of one-eigth of cholesterol (Table VI). When the flocculation was tested by this method, the normal sera showed almost no flocculation in the whole range of serum dilution, whereas flocculations were still found in some zone of concentrations of the immune sera and there was the optimal proportion between the quantities of the sera and cholesterol suspension. This flocculation is regarded as a specific immune reaction. Therefore under this condition the flocculation was examined.—To 0.25 ml. of various dilutions of sera 0.25 ml. of water, 0.25 ml. of 1/160 per cent gelatin solution and 0.25 ml. of 1/20 per cent cholesterol suspension were

TABLE VI

Flocculation of Normal and Immune Sera with and without Addition of Gelatin

(A) To 0.25 ml. of various dilutions of sera 0.5 ml. of water and 0.25 ml. of various dilutions of cholesterol suspension were added, and the mixture was allowed to stand for 24 hours at room temperature.

(B) Instead of 0.5 ml. of water in the above experiment, 0.25 ml. of water and 0.25 ml. of gelatin solution of one-eighth concentration of cholesterol were added.

	Concentration of cholesterol	Concentration of gelatin	Dilution of serum (1 :)									
			2.5	5	10	20	40	80	160	320	640	1280
Normal serum No. 184	%	%	(A)									
	1/5	—	0	0	0	0	0	0	2	3	4	4
	1/10	—	0	0	0	0	0	0		2	3	4
	1/20	—	0	0	0	0	0	0	0	0	1	3
	1/40	—	0	0	0	0	0	0	0	0	0	2
	1/80	—	0	0	0	0	0	0	0	0	0	1
	1/160	—	0	0	0	0	0	0	0	0	0	0
			(B)									
	1/5	1/40	1	0	0	0	0	0	0	0	0	0
	1/20	1/160	0	0	0	0	0	0	0	0	0	0
Immune serum No. 184			(A)									
	1/5	—	4	4	4	3	2	3	4	4	4	4
	1/10	—	3	4	4	4	3	2	3	4	4	4
	1/20	—	2	3	4	4	4	3	2	4	4	4
	1/40	—	0	0	1	2	3	3	3	2	4	4
	1/80	—	0	0	1	2	2	3	2	2	2	4
	1/160	—	0	0	0	0	1	2	2	2	2	2
			(B)									
	1/5	1/40	4	4*	4	3	2	0	0	0	0	0
	1/10	1/80	3	4	4*	4	3	1	0	0	0	0
	1/20	1/160	2	3	3	4*	3	3	2	0	0	0
	1/40	1/320	0	0	2	2	3*	2	2	1	0	0
	1/80	1/640	0	0	1	2	2	3*	2	1	0	0
	1/160	1/1280	0	0	0	0	1	1	2*	1	1	0

* Flocculation appeared at first in these tubes.

Table VII

Flocculation Tests with Cholesterol with the Addition of Gelatin as a Stabilizer

(The same procedures of experiments as Table VI B)

Injections made with	Serum No.		Dilution of serum								No serum
			2.5	5	10	20	40	80	160	320	
Cholesterol and swine serum	184	B	0	0	0	0	0	0	0	0	0
		A	0	2	3	4	3	2	2	1	0
	185	B	0	0	0	0	0	0	0	0	0
		A	3	3	3	3	2	0	0	0	0
	186	B	2	0	0	0	0	0	0	0	0
		A	2	2	1	0	0	0	0	0	0
	187	B	2	1	0	0	0	0	0	0	0
		A	3	3	3	2	1	0	0	0	0
	188	B	0	0	0	0	0	0	0	0	0
		A	3	3	3	2	2	1	1	0	0
	189	B	3	1	0	0	0	0	0	0	0
		A	4	4	4	4	4	3	1	0	0
	190	B	0	0	0	0	0	0	0	0	0
		A	3	4	4	4	3	3	2	1	0
	192	B	3	2	0	0	0	0	0	0	0
		A	3	3	4	3	2	1	0	0	0
	193	B	3	3	2	0	0	0	0	0	0
		A	3	3	3	3	2	1	1	1	0
Cholesterol	194–6	B	0	0	0	0	0	0	0	0	0
		A	0	0	0	0	0	0	0	0	0
	197	B	2	1	0	0	0	0	0	0	0
		A	2	1	0	0	0	0	0	0	0
Swine serum	165–6	B	0	0	0	0	0	0	0	0	0
	172	A	0	0	0	0	0	0	0	0	0

added, and after 24 hours at room temperature (higher than 10°C.) the flocculation was read. The result was: every serum from the rabbits inoculated with the mixtures of cholesterol and swine serum showed positive flocculation, while the serum from those inoculated with either cholesterol or swine serum alone showed no flocculation (TABLE VII).

6. *Complement Fixation of various Kinds of commertially prepared Cholesterol*—The complement fixation was tested using various kinds of commertially prepared cholesterol (Merck, Kahlbaum, Takeda) as antigen, and the results were compared with the reaction of the recrystallized preparation of cholesterol (Takeda). All of them showed almost the same intensity of complement fixation with the above immune sera (TABLE VIII).

TABLE VIII

Complement Fixation with Commertially prepared Cholesterol

The same procedures of experiments as Table III.
Immune serum no. 192

Cholesterol used for antigen	Complement units								
	2	3	4	5	6	7	8	9	10
Takeda, recrystallized	4	4	4	4	3	3	3	2	2
Merck	4	4	4	4	3	3	2	2	2
Kahlbaum	4	4	4	4	3	3	3	2	2
Takeda	4	4	4	4	3	2	2	2	1
Takeda's Laboratory*	4	4	4	4	3	3	2	2	2

* Kindly supplied by Dr. S. Kuwada.

7. *Complement Fixation and Flocculation with Dihydrocholesterole, Cholesterol dibromide, and Cholesterol acetate*—The dihydrocholesterol, cholesterol dibromide and cholesterol acetate had no activity of complement fixation with the above antisera. If cholesterol was reprepared from cholesterol dibromide or cholesterol acetate, it regained the antigenic activity (TABLE IX). The results of the flocculation tests were also the same (TABLE X). From these observations it can be concluded that the antigenic activity of the cholesterol crystals is not due to

their contaminated substances, and their antigenity depends strictly upon their chemical structure.

Table IX

Complement Fixation with Cholesterol Derivatives and purified Cholesterol derived from them

The same procedures of experiments as Table III. Controls (no complement or no serum) showed no fixation.

Antigen	Antiserum no.	Complement units				
		2	4	6	8	10
Recrystallized cholesterol	189	4	3	2	2	1
	190	4	3	2	1	0
	192	4	3	2	1	0
Dihydrocholesterol	189	1	0	0	0	0
	192	1	0	0	0	0
Cholesterol dibromide	189	0	0	0	0	0
	190	0	0	0	0	0
	192	0	0	0	0	0
Cholesterol reprepared from dibromide	189	4	3	2	1	0
	190	4	3	2	2	1
	192	4	3	2	1	0
Cholesterol acetate	189	0	0	0	0	0
	190	0	0	0	0	0
	192	0	0	0	0	0
Cholesterol reprepared from acetate	189	4	3	1	0	0
	190	4	3	1	0	0
	192	4	3	2	0	0

Discussion

As many investigators reported, cholesterol has an anticomplementary action, but it can be eliminated at the temperatures below 15°C. Sachs and Klopstock (1925) (1) and other investigators practised the complement fixation in a refrigerator, but, in order to avoid the anticomplementary action, it is sufficient to keep the reaction temperature at 15°C. Anticomplementary action is somewhat increased in the sera of the rabbits inoculated with the mixtures of

TABLE X

Flocculation Tests with Cholesterol Derivatives and purified Cholesterol derived from them

The same procedures of experiments as Table VI B.
Antiserum no. 184.

Antigen	Dilutions of Antiserum 2.5	5	10	20	40	80	160	320	640
Recrystallized Cholesterol	0	2	3	4	3	2	2	0	0
Cholesterol dibromide	0	0	0	0	0	0	0	0	0
Cholesterol reprepared from dibromide	1	2	3	4	3	2	1	0	0
Cholesterol acetate	1	0	0	0	0	0	0	0	0
Cholesterol reprepared from acetate	1	2	3	4	4	3	2	1	0

cholesterol and swine serum, but it is of far lower degree than the specific complement fixation, and, as Hahn and Hazato (1936) (17) state, the increase of the anticomplementary action is not parallel to the complement fixation activity. The active immune serum can be obtained only when rabbits are inoculated with the mixtures of cholesterol and swine serum. Cholesterol alone can not produce antiserum. The antiserum reacts specific with cholesterol. Some derivatives of cholesterol, namely, dihydrocholesterol, cholesterol dibromide and cholesterol acetate failed to react with the antiserum. These results coincide with the results of Weil and Besser (1931, 1932). (6) If cholesterol was reprepared from these derivatives, it regained the antigenic activity, and these procedures of purification did not weaken the antigenity of the cholesterol. All these facts indicate that cholesterol has antigenic activity when injection is made with swine serum as socalled "Schlepper", and its activity does not depend on its contaminated substances. Wadsworth, Maltaner and Maltaner (1935)(16) reported that the obtained sera showed only increased anticomplementary activity and not the specific complement fixation, but the conclusion was not deduced from the sufficient active immune sera. The above observation can distinctly distinguish the specific complement fixation from the increased anticomplementary activity. Furthermore, the specific activity of immune sera can be proved also by flocculation tests, as Weil and den Dooren de Jong (1937) (7) reported. In this case the non-specific precipitation of cholesterol by the dilution of the sera with physiological saline can be eliminated by adding gelatin solution as a protective colloid.

SUMMARY

The rabbits treated with the mixture of cholesterol and swine serum were proved to produce the antiserum reacting specific with cholesterol by complement fixation and flocculation tests. The anticomplementary action of cholesterol can be eliminated by the low reaction temperature, and the effect of the instability of the colloidal state of cholesterol can be eliminated by adding gelatin solution as a stabilizer. The purification of cholesterol does not weaken its antigenic activity. Dihydrocholesterol, cholesterol dibromide and cholesterol acetate do not react with this antiserum.

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ON THE PROTEASE ACTION OF PENICILLIUM NOTATUM. I.

By

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Considerable works have been done on the proteolytic actions of molds. Concerning *Aspergilli* molds, Malfitano (1) recognized the gelatin-splitting activity of *Asp. niger* and Otani (2) reported also on the same theme. As to the proteolytic actions of *Asp. oryzae*, investigations were done by Sumi (3) and also by Nishikawa (4) who affirmed the presence of tryptase activity (about pH 8), but did not acknowledge that of pepsin activity. In the mean time, it was reported by Oshima (5) and Otani (6) that the proteinase of *Aps. oryzae* belongs to papainase, while the report of Stakhecva-Kaverzneva *etc.* (7) indicated that the optimum pH for gelatin and collagen-splitting activity of the *oryzae* enzyme lies at pH 7.2.

As regards the peptidase activity, various observations on *Asp. niger* were made by Abderhalden *et al.* (8) and Schmalfuss (9), who also found the diglycine-splitting activity of *Asp. niger.*

Since the first discovery in 1881 of what we call histozyme in a pig's kidney by Schmiedberg (10), its existence has been proved in various organs of many animals. As to the molds, the histozyme was found in *Asp. niger* by Shibata (11) and in *Asp. oryzae* by Neuberg *et al.* (12). Otani (13) has succeeded to ascertain the splitting-ability of various species of molds on the benzoyl-derivatives of various peptides. Notwithstanding these numerous observations so far made on the proteolytic actions of molds, very few of them have been carried out by the recent classification of the protease. Johnson and his coworkers (14, 15) described that the proteolytic system of *Asp. parasiticus* consists of at least four components; namely, a proteinase, a carboxypolypeptidase, an aminopolypeptidase and a dipeptidase. As to the proteinase action, the optimum pH for the hydrolysis of gelatin was found to be about pH 7.3 and the action was inactivated by egg albumin, but was activated neither by animal enterokinase, H_2S nor by HCN. Their further investigations as to the contents of proteolytic enzymes of thirty common molds (16) lead them to conclude that the proteolytic system consists of at least one proteinase and five peptidases; namely, a dipeptidase, a carboxypolypeptidase, an aminopolypeptidase and two enzymes that hydrolyse diglycine and triglycine. The optimum reaction for the proteinase of three species of *Aspergilli* acting on gelatin was found to be about pH 7.

As far as the *Penicillia* enzyme is concerned, Dox (17) recognized already in 1909 the proteolytic activity in *Pen. camemberti* as well as in *Pen. chrysogenum*; many years after that, Yamaguchi (18) found that the proteolytic system of *Pen. glaucum* contains pepsin, trypsin and erepsin, and Wada (19) also reported on the existence of trypsin as well as erepsin-like exzymes in the mold of the same species. But none of these works have been done by the systematical method adopted at present. In 1940, a report on the presence of the proteolytic enzyme in *Penicillium* was filed by Kuprevich (20). These observations suggest the presence of tryptase, pepsin or papainase in *Penicillia*, and there remains an interesting problem in the field of comparative enzymology. Recently, Thibodeau *et al.* (21) have reported that *Pen. roqueforti* appeared to produce only one protease, which had its optimum activity between 5.8 and 6.3 on casein and that the protease might be of a trypsin type but not identical with the animal trypsin. It is now very desirable to throw light on the characteristics of the *Penicillium* protease. Since the *Penicillium* can be produced in a great deal nowadays, it is our understanding that it will make a great contribution not only to the enzymology but also to its utilization, if we can give a fundamental knowledge of its proteolytic actions.

The present paper reports on the results obtained from the studies on the protease actions of *Pen. notatum.*

EXPERIMENTAL

TABLE I

Optimal pH for proteinase and peptonase of *Pen. notatum* (Q 176).

Enzyme: *Pen. notatum,* P 176, cultivated on Czapeck-Dox medium added with 5% peptone and Q 176 strain of that cultivated on similar medium for 7 days were used as enzyme sources. The mycelium, which was washed repeatedly with physiological NaCl solution, was ground in a mortar with 2 times volume of glycerine-water (1: 1) and was filtered through gauze. The maceration thus obtained was kept in ice-box under layers of toluene and was used as an enzyme source.

Test procedure: 5 ml. 4% substrate solution+5 ml. enzyme solution +10 ml. buffer solution of various pH values+toluene; incubated at 37°C. Control solution, containing only enzyme and buffer without substrate, was treated in just the same way.

After a definite time, the acidity increase in 4 ml. digestate was measured by the formol titration with 0.1 *N* NaOH solution. The values obtained from the control experiments were deducted from those of the main experiments, and the corrected values of acidity increase are shown in the table.

Acidity increase in 4 ml. digestate (ml. 0.1 *N* NaOH)

Substrate	pH / Time hrs.	4.0	5.0	5.6	5.8	6.0	6.2	6.4	6.6	6.8	7.0	7.5	8.0	9.0
Gelatin	5	0	0.15	—	0.20	0.21	0.26	0.27	**0.32**	**0.35**	**0.31**	0.22	0.21	0.05
	24	0	0.46	—	0.63	0.63	0.67	0.71	**0.83**	**0.78**	0.70	0.68	0.63	0.25
	72	0	0.75	—	1.12	1.11	1.15	1.25	**1.33**	**1.30**	1.25	0.99	0.91	0.44
Casein (Hammarsten)	5	0.15	0.65	**1.01**	**1.10**	**1.03**	0.88	0.76	0.36	—	0.45	0.38	0.31	0.21
	24	0.47	1.03	**1.95**	**1.72**	**1.73**	1.50	1.21	0.44	—	0.63	0.67	0.41	0.21
	72	0.67	1.12	**2.14**	**1.77**	**1.80**	1.73	1.49	0.52	—	0.82	0.73	0.45	0.21
Peptone (Riedel)	5	0	0.45	0.67	—	0.83	**0.89**	**0.91**	**0.91**	**0.91**	**0.88**	0.80	0.60	0.50
	24	0	0.89	1.25	—	**1.34**	**1.36**	**1.36**	**1.37**	**1.34**	1.21	1.04	0.79	0.64
	72	0	1.19	1.38	—	1.45	**1.51**	**1.51**	**1.51**	**1.52**	1.38	1.14	0.79	0.64

TABLE II.

Protease action of maceration and acetone powder (P 176).

Enzyme: Maceration, prepared with 4 times volume of glycerine-water (1 : 1), and 2% water suspension of acetone powder were employed.

The ground mycelium was treated with 3 times volume of acetone and was centrifuged after stirring. The operation was repeated times, and then it was treated with ether. It was filtered by suction and was dried over H_2SO_4 in vacuum desiccator after evaporation of ether. The acetone powder thus obtained was used as 2% water suspension in the experiments.

Test procedure: 5 ml. 4% substrate solution + 2 ml. maceration or 2% water suspension of acetone powder + 13 ml. buffer solution of various pH values + toluene; incubated at 37°C.

Acidity increase in 4 ml. digestate (ml. 0.1 *N* NaOH)

Substrate	Enzyme	pH / Time hrs.	4.0	5.0	6.0	6.5	7.0	8.0	9.0
Gelatin	Maceration	24	0	0.08	0.11	**0.13**	**0.16**	**0.14**	0.10
		72	0	0.16	0.30	**0.34**	**0.37**	0.15	0.12
		120	0	0.23	0.41	**0.58**	**0.55**	0.25	0.14
	Powder	24	0	0.05	0.04	**0.09**	**0.08**	0.04	0
		72	0	0.06	0.14	**0.21**	**0.19**	0.06	0
		120	0	0.08	0.24	**0.34**	**0.27**	0.17	0
Casein	Maceration	24	0	0.25	**1.22**	**1.19**	0.83	0.25	—
		72	0	0.66	**2.15**	**2.10**	1.63	0.67	—
		120	0	0.79	**2.46**	**2.49**	1.90	0.92	—
	Powder	24	0	0.13	**0.22**	**0.21**	**0.21**	0.09	0.09
		72	0	0.19	**0.49**	**0.49**	**0.46**	0.20	0.14
		120	0	0.39	**0.68**	**0.69**	**0.66**	0.26	0.24
Peptone	Maceration ×	24	0	0	**0.22**	**0.22**	**0.22**	0	0
		72	0	0.21	**0.39**	**0.34**	**0.34**	0	0
		120	0	0.22	**0.39**	**0.35**	**0.35**	0	0
	Powder △	24	0	0.24	**0.40**	**0.49**	**0.53**	**0.47**	0.37
		72	0	0.37	**0.74**	**0.73**	**0.80**	0.54	0.51
		120	0	0.48	**0.89**	**0.91**	**0.91**	0.59	0.51

× ....Peptone U.   △............Peptone Riedel

TABLE III

Influence of H_2S on the proteolytic action of *Pen. notatum* (P 176).

Enzyme: Meceration prepared with 3 times volume of glycerine-water (1 : 1).

Test procedure: 5 ml. 4% substrate solution + 2 ml. maceration + 13 ml. buffer solution of various pH values + toluene; incubated at 37°C. The enzyme solution was preliminary saturated with H_2S for 30 minutes at room temperature. In the control experiments, the enzyme solution was used as it was without H_2S.

Acidity increase in 4 ml. digestate (ml. 0.1 *N* NaOH)

Substrate	Experiment	pH Time hrs.	4.0	5.0	6.0	6.5	7.0	7.5	8.0	9.0
Gelatin	a) Without H_2S	24	0	0.06	0.10	0.13	0.16	0.13	0.13	0.11
		72	0	0.15	0.26	0.34	0.34	0.27	0.28	0.12
	b) With H_2S	24	0	0.06	0.10	0.13	0.16	0.13	0.13	0.11
		72	0	0.15	0.26	0.34	0.34	0.27	0.28	0.12
	Activation (b-a)	24	0	0	0	0	0	0	0	0
		72	0	0	0	0	0	0	0	0
Casein	a) Without H_2S	24	0	0.20	0.25	0.19	0.29	0.28	0.18	0.13
		72	0	0.32	1.08	1.83	0.94	0.91	0.68	0.44
	b) With H_2S	24	0	0.03	0.19	0.10	0.17	0.19	0.18	0.13
		72	0	0.12	0.77	0.63	0.74	0.66	0.68	0.44
	Activation (b-a)	24	0	−0.17	−0.06	−0.09	−0.12	−0.09	0	0
		72	0	−0.20	−0.31	−0.20	−0.20	−0.25	0	0
Peptone (U.)	a) Without H_2S	24	0	0.20	0.22	0.17	0.26	0.17	0.13	0
		72	0	0.35	0.36	0.33	0.33	0.28	0.20	0
	b) With H_2S	24	0	0.17	0.16	0.16	0.23	0.19	0.15	0
		72	0	0.39	0.36	0.31	0.33	0.28	0.19	0
	Activation (b-a)	24	0	−0.03	−0.03	−0.01	−0.03	−0.02	−0.02	0
		72	0	+0.04	0	−0.02	− 0	0	−0.01	0

TABLE IV

Influence of cysteine on the proteolytic action of ***Pen. notatum*** (P 176).

Enzyme: Maceration prepared with 2 times volume of glycerine-water (1: 1).

Test procedure: 5 ml. 4% substrate solution + 2 ml. enzyme solution + 2 ml. 0.1 M cysteine-HCl solution or distilled water instead of activator (control) + 11 ml. buffer solution of various pH values + toluene; incubated at 37°C. Enzyme-cysteine-HCl mixtures and enzyme-water mixtures (controls) were adjusted to pH 6.0, incubated at 37°C for 30 minutes and then tested in the experiments.

Acidity increase in 4 ml. digestate (ml. 0.1 N NaOH)								
Substrate	Experiment	Time hrs. \ pH	4.0	5.0	6.0	7.0	8.0	9.0
Gelatin	a) Without cysteine	24	0.13	0.29	0.43	0.47	0.38	0.30
		72	0.15	0.44	0.83	0.83	0.44	0.39
	b) With cysteine	24	0.13	0.29	0.43	0.32	0.25	0.30
		72	0.15	0.44	0.67	0.73	0.44	0.27
	Activation (b-a)	24	0	0	0	−0.15	−0.13	0
		72	0	0	−0.16	−0.10	0	−0.12
Casein (Hammarsten)	a) Without cysteine	24	0	0.27	0.52	0.41	0.12	0.12
		72	0	0.49	1.26	1.04	0.24	0.20
	b) With cysteine	24	0	0.27	0.52	0.41	0.20	0.12
		72	0	0.49	1.26	1.04	0.40	0.20
	Activation (b-a)	24	0	0	0	0	+0.08	0
		72	0	0	0	0	+0.16	0

TABLE V

Influence of KCN on the protease action of ***Pen. notatum*** (Q 176).

Enzyme: Maceration prepared with 2 times volume of glycerine-water (1: 1).

Test procedure: 5 ml. 4% substrate solution 5 ml. maceration + 2 ml. 0.1 M KCN solution or distilled water instead of activator (control) + 8 ml. buffer solution of various pH values + toluene; incubated at 37° C.

Enzyme-KCN mixtures and enzyme-water mixtures (controls) were adjusted to pH 6.0, incubated at 37°C for 30 minutes and used.

Acidity increase in 4 ml. digestate (ml. 0.1 *N* NaOH)

Substrate	Experiment	pH Time hrs.	4.0	5.0	6.0	7.0	8.0	9.0
Gelatin	a) Without KCN	5	0	0.06	0.22	0.32	0.20	0.16
		24	0	0.40	0.66	0.82	0.46	0.32
		72	0	0.76	1.25	1.27	0.63	0.47
	b) With KCN	5	0	0.12	0.17	0.32	0.20	0.16
		24	0	0.40	0.63	0.82	0.46	0.32
		72	0	0.74	1.24	1.27	0.63	0.47
	Activation (b-a)	5	0	+0.06	−0.05	0	0	0
		24	0	0	−0.03	0	0	0
		72	0	−0.02	−0.01	0	0	0
Casein (Hammarsten)	a) Without KCN	5	0	0.28	0.69	0.50	0.36	0.20
		24	0	0.66	1.35	0.99	0.71	0.53
		72	0	1.04	1.51	1.10	0.77	0.61
	b) With KCN	5	0	0.28	0.69	0.50	0.36	0.20
		24	0	0.66	1.35	1.00	0.70	0.53
		72	0	1.04	1.51	1.10	0.77	0.61
	Activation (b-a)	5	0	0	0	0	0	0
		24	0	0	0	+0.01	−0.01	0
		72	0	0	0	0	0	0
Peptone (Riedel)	a) Without KCN	5	0	0.24	0.55	0.56	0.40	0.26
		24	0	0.74	1.16	1.15	0.80	0.60
		72	0	1.02	1.30	1.26	0.80	0.60
	b) With KCN	5	0	0.24	0.55	0.56	0.40	0.26
		24	0	0.74	1.16	1.15	0.08	0.60
		72	0	1.02	1.30	1.26	0.80	0.60
	Activation (b-a)	5	0	0	0	0	0	0
		24	0	0	0	0	0	0
		72	0	0	0	0	0	0

TABLE VI

Inhibiting action of egg albumin on the proteinase action of *Pen. notatum* (Q 176) at pH 6.8.

Enzyme: Maceration was prepared with 2 times volume of glycerine-water (1 : 1).

Test procedure: 5 ml. 4% gelatin solution+5 ml. maceration+2 ml. egg albumin solution of various % or distilled water (control)+8 ml. buffer solution (pH 6.8)+toluene; incubated at 37°C.

The final concentration of egg albumin (E. Merck) in test mixtures is shown in the table.

The values of the control experiments without albumin were reduced from those of the main experiments with albumin and these corrected values are given in the parenthesis in the table.

Acidity increase in 4 ml. digestate (ml. 0.1 *N* NaOH)

Time hrs.	Concentration of albumin						
	2%	1%	0.5%	0.2%	0.1%	0.05%	0
24	0 (−0.55)	0.03 (−0.52)	0.01 (−0.54)	0.05 (−0.50)	0.01 (−0.44)	0.02 (−0.53)	0.55
72	0.05 (−0.59)	0.09 (−0.55)	0.02 (−0.62)	0.07 (−0.57)	0.16 (−0.48)	0.13 (−0.51)	0.64
120	0.06 (−0.69)	0.10 (−0.65)	0.08 (−0.67)	0.12 (−0.63)	0.16 (−0.59)	0.13 (−0.62)	0.75

Time hrs.	Concentration of albumin						
	0.05%	0.02%	0.01%	0.005%	0.002%	0.001%	0
24	0 (−0.27)	0.16 (−0.11)	0.26 (−0.11)	0.21 (−0.06)	0.36 (−0.01)	0.27 (0)	0.27
72	0.06 (−0.45)	0.23 (−0.28)	0.39 (−0.12)	0.40 (−0.11)	0.40 (−0.11)	0.51 (0)	0.51
120	0.13 (−0.53)	0.35 (−0.31)	0.48 (−0.18)	0.53 (−0.13)	0.55 (−0.11)	0.66 (0)	0.66

Table VII

Hydrolysis grade of various proteins by the proteinase of *Pen notatum* (P 176) at pH 7.0.

Enzyme: The maceration prepared with 2 times volume of glycerine-water (1 : 1) and the 4% water suspension of acetone powder were tested.

Test procedure: 5 ml. 4% protein solution + 2 ml. maceration or 4% powder suspension + 13 ml. buffer solution of pH 7.0 + toluene; incubated at 37°C.

Acidity increase in 4 ml. digestate (ml. 0.1 N NaOH)

Enzyme	Substrate / Time hrs.	Casein (Hammarsten)	Fibrin	Gelatin	Egg albumin (E. Merck)	Edestin
Maceration	24	1.37	0.55	0.56	0.26	0.24
	72	1.85	1.04	0.97	0.41	0.29
	120	1.92	1.22	1.14	0.48	0.33
Acetone powder	24	0.39	0.14	0.02	0	0
	72	0.84	0.61	0.21	0	0
	120	1.53	0.78	0.32	0	0

Table VIII

Distribution of the proteinase (gelatinase) of *notatum* (Q 176) maceration after incubation at 37°C.

Enzyme: The maceration prepared with 2 times volume of glycerine-water (1 : 1) was allowed to stand at 37°C for a definite period under toluene. It was then centrifuged and separated into a supernatant liquid and a sediment. The sediment was made up to the original amount by addition of glycerine-water (1 : 1). Besides the whole autolysate mixture, these two components were tested as enzyme sources in the experiments.

Test procedure: 5 ml. 4% gelatin solution + 5 ml. enzyme solution + 10 ml. buffer solution of pH 6.8 + toluene; incubated at 37°C.

Acidity increase in 4 ml. digestate (ml. 0.1 N NaOH)

Days of incubation	Enzyme / Time hrs.	Whole autolysate	Supernatant liquid	Sediment suspension
0	5	0.26	0.27	0.08
	24	0.64	0.65	0.24
	72	0.88	0.87	0.38
1	5	0.24	0.24	0.07
	24	0.62	0.62	0.25
	72	0.89	0.88	0.40
6	5	0.19	0.20	0.06
	24	0.51	0.48	0.14
	72	0.80	0.77	0.39
14	5	0.18	0.17	0
	24	0.24	0.42	0.10
	72	0.65	0.65	0.13

RESULTS AND CONSIDERATIONS

The proteolytic system of the maceration of *notatum* mycelium as well as the acetone powder prepared by means of acetone-ether method was examined in the experiments.

Gelatin, casein and peptone were hydrolysed both by the maceration and the acetone powder between pH 5.0 and 9.0 and the optimum reaction lies neutral or at a weak acid side. The maceration is excellent in cleavability of these substrates, compared with the acetone powder (TABLE I and II). In case of peptones, peptone (Riedel) was attacked more easily than peptone (Uehara, Teruuchi *etc.*), which seems to contain more lower moleculs than the former. Although the maceration was used in the experiments of the latter, the hydrolysis value was not remarkable. After five hour incubation at 37°C, it resulted that the maximum value of gelatin-hydrolysis occurred at pH 6.8, that of casein at pH 5.8 and that of peptone at pH 6.4—6.8 (Table I). The optimum pH for the casein hydrolysis was found to be at a little acid side than that of gelatin and peptone. It can be seen from these results that the optimum pH for the gelatin hydrolysis is similar to the reports of Stakheeva-Kaverzneva *et al.* (7) and Johnson *et al.* (14, 15, 16) about *Aspergilli*, and that of casein splitting coincides with the investigation of Thibodeau *et al.* (21), consequently in every case the optimum pH inclining towards a little acid side from the neutral reaction.

It must be here cited that the optimum pH for proteinase of common bacteria lies at alkaline side and that of plant proteinase at acid side. As the plant acid proteinase is activated by H_2S, cysteine and HCN, it is very interesting to investigate into what sort of influences should be given by these substances upon the proteolytic actions of *Pen. notatum.* Table III and IV indicate that the hydrolysis of gelatin, casein and peptone by the maceration is neither stimulated nor inhibited especially by addition of H_2S, cysteine and KCN, both in acid and alkaline reaction. On the contrary, the enzyme is inhibited by egg albumin in a concentration of more than 0.02 per cent, as Table VI shows. Accordingly, even if this enzyme seems to denote a catheptic nature from the point of optimum pH, it must be distinguished from the catheptase (papainase), because of its non-activability by H_2S, cysteine and KCN, and of its inhibitability by albumin, as is seen in case of pancreatic trypsin. The results obtained from the experiments concerning the problems of activation and inhibition coincide perfectly with the report of Johnson (14) about *Asp. parasiticus.* These observations lead to the conclusion that the proteinase and peptonase of *Pen. notatum* are quite different from the well-known proteinase of animal as well as higher plant.

The results of investigations then carried out about the cleavability of various proteins by the enzyme, showed that the relative rates of hydrolysis by maceration at pH 7.0 diminished in the following order (Table VII).

$$\text{Casein} > \text{fibrin} \geqq \text{gelatin} > \text{albumin} \geqq \text{edestin}$$

Casein is the most digestable protein, fibrin and gelatin are the next and albumin as well as edestin are the least, being not attacked by acetone powder.

The distribution of proteinase in the *notatum* maceration was then studied. It can be understood from Table VIII that the greater part of the proteinase of the maceration moves into the supernatant liquid in a short time and scarcely remains in the sediment. The results is coincident with the observation of Johnson (14) about *Asp. parasiticus* that the proteinase is an exoenzyme, contrary to the peptidase, which is not yet examined in the present case. When incubating the maceration of *Pen. notatum* at 37°C, the proteinase is very resistant to it and has scarcely diminished its activity after two weeks. But the activity was hardly recognized in the sediment of autolysed maceration. This result may be considered to be due to the resolution of the enzyme into the liquid according to the advancement of autolysis.

SUMMARY

1) Both maceration and acetone powder of *Pen. notatum* hydrolyse gelatin, casein and peptone at a pH range of 5.0 to 9.0. The optimum pH for the hydrolysis was found to be at pH 6.8 for gelatin, at pH 5.8 for casein and from pH 6.4 to 6.8 for peptone.

2) The acetone powder prepared by the ordinary acetone-ether method has also each proteinase activity, but it seems to undergo partial inactivation.

3) This proteolytic activity is neither accelerated nor inhibited by addition of H_2S, cysteine and KCN both in acid and alkaline reaction. But the proteinase is remarkably inhibited by egg albumin in a concentration of more than 0.02 per cent at pH 6.8.

4) The degree of hydrolysis of various proteins at pH 7.0 diminished in the following order.

$$\text{Casein} > \text{fibrin} \geqq \text{gelatin} > \text{albumin} \geqq \text{edestin}$$

Albumin and edestin were attacked even in a small quantity by the maceration, but never by the acetone powder.

5) Proteinase of *Pen. notatum* can be easily extracted with glycerine-water. It is very resistant to keeping at 37°C and drops scarcely its activity even after two weeks.

In closing I wish to express my deepest gratitude to Dr. S. Utzino, Prof. at the Department of Medicine, Kyoto University, for his kind constant guidance in this research.

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β-CARBOXYLASE MODEL*

By

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The following article is the outline of the studies in this institute during the war-time on the catalytic decarboxylation of β-carboxylic acid.

According to Kaneko (1938) amino-antipyrine could decompose acetacetic acid into acetone and CO_2 very rapidly (pH optimum 4.2) and nearly 80% CO_2 were produced in 5 minutes. Kuga (1942) remarked the presence of acetacetic acid residue in the amino-antipyrine molecule and found as another catalyst 4-methyl-5-amino-pyrimidine, which is an amino derivative of acetacetic acid-urea condensation product. Fukuda (1943) ascertained subsequently 2-methyl-3-amino-4-hydroxy-quinoline was catalytically active, which is an amino derivative of acetacetic acid-aniline condensation product.

These compounds have each a free amino group in the α-C position of the acetacetic acid residue, β-C atom of which combines on the other hand with N of another molecular part. The free amino group is the active center. If it is previously methylated, catalysis can never be seen; pyramidone is inactive. The structural analogy of that residue to acetacetic acid should favour the action of the amino group. The mechanism may be an increase of affinity between the catalyst and the substrate or an induced deformation of back bone of the substrate molecule. At any rate through combination of β-keto group of acetacetic acid with the amino group of the catalyst, the carboxyl of the substrate becomes unstable, decarboxylation occurs and the remaining Schiff's base either dissociates spontaneously to reproduce the original free catalyst or reacts as Langenbeck (1933) said successively with a second molecule of acetacetic acid under acetone liberation.

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* A part of contents of "the specificity of enzyme action", spoken at the 12th General Japanese Medical Congress, 3 April 1947, Osaka Municipal Auditorium, Osaka.

The relation of chemical constitution of β-carboxylase models to their catalytic power seems very interesting in viewpoint of the specificity of enzyme action.

Beniya (1934) had already observed that ethylene diamine and o-phenylen diamine decarboxylate β-keto acid more intensively than ethylamine resp. aniline, but either m- or p-phenylendiamine is only so scarcely active as aniline. Hence the second amino group in neighbouring position should intensify the decarboxylating activity. Fukuda (1943) and Kamiya (1947) observed indeed that 4, 5-diamino-uracil and 2, 3-diamino-4-hydroxy-quinoline are more active than 4-methyl-5-amino-uracil resp. 2-methyl-3-amino-4-hydroxy-quinoline. Therefore a diamino derivative of pyrazolone might be more active than amino-antipyrine. Kamiya (1947) observed 1-phenyl-4-amino-5-methyl-pyrazolone (3) is able to decarboxylate acetacetic acid almost in the same velocity as amino-antipyrine does. He synthesized then a diamino derivative of that another type pyrazolone, 1-phenyl-3-hydroxy-4-amino-pyrazolone (5)-imide. This is the most active amino compound we have ever assayed, but as a β-carboxylase model it is less adequate than amino-antipyrine on account of its spontaneous oxidability.

In the molecules of those heterocyclic diamino compounds there is no more acetacetic acid residue, because methyl has been exchanged with an another amino group. But we know according to Grimm's law that amino group behaves electrically in the same manner as methyl group.

Amino-oxindol is one of the intensive catalysts in case of α-keto acid decarboxylation. This Langenbeck's enzyme model (1933) is scarcely active to acetacetic acid, perhaps owing to the absence of β-keto acid residue. But on account of the result of Fukuda

(1943) that 4-methyl-5-amino-uracil was more effective than amino-malonyl-guanidine, 2-methyl-3-amino-indol should be also more active than amino-oxindol. Indeed its activity was of the same degree as that of o-phenylen diamine, though it was inactive to pyruvic acid. We have not synthesized 2, 3-diamino-indol, but this compound might be too autoxidable to be used for the experiment.

The β-barboxylase model must have as above mentioned a free group and moreover a structually substrate-analogous residue for enhancement of activity. But the degree of activity is on the other hand dependent of the another part of catalyst molecules. Regular variation of catalytic power through the sorts of heterocyclic nucleus and substituting group is evident in the following table, to which the results with pyridine derivatives are also added. 2, 3-diamino-6-methyl-pyridine was not examined. If it were synthesized, it should be ordered at the supposed position.

Table I

1-Phenyl-3-hydroxy-4-amino-pyrazolone (5)-imide	>	Amino-antipyrine & 1-Phenyl-4-amino-5-methyl pyrazolone (3)	>	1-Phenyl-3-methyl-4-amino-pyrazolone
V		V		V
4, 5-Diamino-uracil	>	4-Methyl-5-amino-uracil	>	2-Methoxy-4-methyl-5-amino-pyrimidine
V‖		V		V
2, 3-Diamino-4-hydroxy-quinoline	>	2-Methyl- 3-amino-4-hydroxy-quinoline	>	2-Methyl-3-amino-quinoline
		V		‖
(2, 3-Diamino-4-hydroxy-6-methyl-pyridine)		2, 6-Dimethyl-6-amino-4-hydroxy-pyridine	>	2, 6-Dimethyl-3-amino-pyridine

The catalytic decarboxylation of acetacetic acid was investigated at first by Widmark (1922). The catalyst was aniline. Its action is nevertheless not to be said now as strong enough. We have assayed several benzene derivatives. They show also a regularly variating activity in relation to their side chains. They are ordered in the next table in comparison with pyrimidine derivatives.

TABLE II

Diamino-uracil	>	Methyl-amino-uracil	>	Amino-uracil	>	Amino-malonyl-guanidine	>	Amino-uracil-carbonic acid
V		V		V		V		V
o-Phenylen-diamine	>	c-Toluidine	>	Aniline	>	o-Amino-phenol	>	Anthranilic acid

EXPERIMENTAL

Catalysts. Most of the assayed amino compounds were prepared in this institute.

I. 4-Methyl-5-amino-uracil: Oranic syntheses. **17**, Behrend and Osten (1905).

II. 5-Amino-uracil: Behrend and Roosen (1889).

III. 4, 5-Diamino-uracil: Traube (1900).

IV. 5-Amino-uracil-4-carbonic acid: Reduction of potassium 5-nitro-uracil-4-carbonic acid according to Bogert and Davidson (1933).

V. Amino-malonyl-guanidine: Traube (1893).

VI. 2-Methyl-3-amino-4-hydroxy-quinoline: Conrad and Limpach (1887).

VII. 2, 3-Diamino-4-hydroxy-quinoline: Gabriel (1918).

VIII. Amino-quinaldine (2-Methyl-3-amino-quinoline): Lawson, Perkin and Robinson (1924).

IX. 1-Phenyl-4-amino-5-methyl-pyrazolone (3): Michaelis and Mayer (1903), Kotelmann (1906).

X. 1-Phenyl-3-hydroxy-4-amino-pyrazolone (5)-imide:

1-Phenyl-3-hydroxy-pyrazolone (5)-imide was prepared according to Conrad and Zart (1906). 4.4 g. of it are dissolved in 2.2 ml. of 1 *N* NaOH, treated with sodium nitrite (1.8 g.) solution and poured into 26 ml. of 20% acetic acid. The brick-red precipitate is washed with water. 1 g. of nitroso compound is dissolved in 12 ml. of 51% HI and 1 g. of powdered phosphonium iodide added gradually under ice cooling. The solution changes from dark brown to red. It is concentrated under diminished pressure, sepapated yellow crystalls of imide iodohydrate are filtered, dissolved in a little water and neutralized with dilute ammoniak. The free imide precipitates in orange-red crystalls of drused form, which are considerably autoxydable and become red brown. They are therefore imediately suspended in 3 ml. of water, neutralized to congo-red with dilute HCl and the filtrate is added with 5 volume of conc. HCl, when colorless plates of imide hydrochloride separate out. Yielded 0.9 g., m. p. 185°. This salt keeps also not long and becomes gradually orange even in vacuum. The compound had the following composition.

$C_9H_{10}O_1N_4 \cdot 2HCl$ Calculated N 21.3%, C 41.1%, H 4.56%
Found N 21.9%, C 42.6%, H 4.73%

XI. Amino-lutidone (2, D-dimethyl-3-amino-4-hydroxy-pyridine); Hall and Collie (1898).

XII. 2-Methoxy-4-methyl-5-amino-pyrimidine and 2, 6-dimethyl-3-amino-pyridine were supplied by Prof. Ochiai, Tokyo University, to whom the author wishes to express his thanks.

XIII. Methyl-Ketol (1-Methyl-2-amino-indol): Methyl-Ketol was prepared according to Fischer (1886). Amination of it was done according to Wagner (1887).

Volume of CO_2 was measured by the Kobayashi's apparatus. 0.5 ml. of $M/10$ catalyst solution + 10 ml. of $M/2$ acetate buffer (pH 4.2) were mixed with 1 ml. of 1 M sodium acetacetate solution. This substrate solution was prepared as follows. 5 ml. of 2 N NaOH were added to 1.09 ml. of acetacetic methylester in 10 ml. volumetric flask, filled with water to the mark and allowed to stand overnight at room temperature. CO_2 evolution was measured every 5 minutes at 37°. 100% decomposition of acetacetic acid should form 25 ml. of CO_2. Control was carried through in the same time using 0.5 ml. of water instead of the catalyst solution for the substraction of the blank from main test.

Table III. *CO_2 formation from 1 ml. of 1 M acetacetic acid at 37°C, pH 4.2 by 1 ml. of the following amino compound solutions.*

Amino compound	CO_2 (ml.)					
	5 min.	10 min.	15 min.	20 min.	25 min.	30 min.
M/10 Amino-antipyrine	17.15	20.85	23.20	23.70	23.95	24.05
M/10 1-Phenyl-4-amino-5-Methyl-pyrazolone (3)	16.80	21.25	23.15	23.75	23.90	23.95
M/10 1-Phenyl-3-hydroxy-4-amino-pyrazolone (5)-imide	20.25	22.25	22.65	23.05	23.20	23.30
M/10 Amino-quinaldine	0.30	0.55	0.65	0.75	—	0.75
M/10 Amino-hydroxy-quinaldine	4.50	7.10	9.10	10.90	12.20	13.70
M/10 Diamino-hydroxy-quinoline	13.60	18.60	21.45	22.70	23.10	23.40
M/10 2-Methoxy-4-methyl-5-amino pyrimidine	0.90	1.40	1.65	1.85	2.25	2.45
M/10 4-Methyl-5-amino-uracil	7.00	11.00	14.55	16.70	17.95	18.46
M/10 4, 5-Diamino-uracil	13.75	20.00	22.13	22.60	—	22.60
M/10 5-Amino-uracil	4.00	5.55	6.65	7.65	8.35	8.85
M/10 Amino-malonyl-guanidine	1.65	2.90	4.05	4.80	5.10	5.35
M/10 5-Amino-uracil carbonic acid	2.00	—	—	2.00	—	2.00
M/10 Amino-oxindol	0.20	0.36	0.30	—	—	0.30
M/10 Amino-methyl-ketol	3.65	6.00	7.90	9.45	10.65	11.50
M/10 β-Amino-$\alpha\alpha$-lutidine	0.30	0.55	0.65	—	—	0.65
M/10 β-Amino-$\alpha\alpha$-lutidone	0.50	0.90	1.40	1.60	2.10	2.20

M/10 o-Phenylen diamine	3.75	6.05	7.60	8.80	9.80	10.80
M/10 o-Toluidine	0.45	0.90	1.35	1.80	2.15	2.40
M/10 Aniline	0.45	0.90	1.25	1.45	1.70	1.90
M/10 o-Amino-phenol	0.35	0.70	1.00	1.25	1.50	1.75
M/10 Anthranilic acid	0.40	0.70	0.75	0.80	0.85	0.90
M o-Toluidine	3.60	6.15	8.10	9.70	11.10	12.10
M Aniline	3.45	5.65	7.65	9.15	10.20	11.20
M o-Amino-phenol	3.15	5.20	6.35	8.05	9.15	10.40
M Anthranilic acid	1.30	1.65	1.85	2.00	2.10	2.20

SUMMARY

Decarboxylation of acetacetic acid by various amino compounds was investigated. Catalytic power depends upon the constitution of the compounds, but the principal condition for the great activity is the presence of acetacetic acid residue in the molecules. Mechanism of action of β-carboxylase model was discussed from viewpoint of specificity of enzyme action.

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THE RÔLE OF NITRATE IN BIOLOGICAL OXIDATIONS. THE EFFECTS OF NITRATE ON CELL RESPIRATIONS

By

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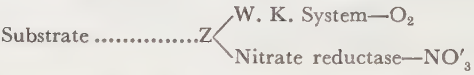
The biological reduction of nitrate to nitrite has been observed in plants, animals and micro-organisms. The physiological meaning of the reduction is self-evident at least in plants, which can use nitrate as a source of nitrogen. But the meaning of this reduction remained obscure until recently in the cases of animals and some micro-organisms.

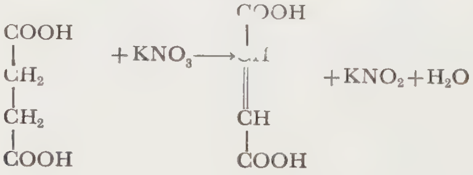
Quastel, Stephenson and Whetham (1) have shown that *Escherichia coli* and some other facultative anaerobes which contain a specific enzyme reducing nitrate are able to grow in the lactate medium in anaerobiosis only in the presence of nitrate. In this case nitrate acts not as a nitrogen source but as an ultimate hydrogen acceptor in place of molecular oxygen. E. Aubel and one (Egami) of us (2) have also shown that in some biological oxidations only nitrate but neither quinone nor methylene blue can replace molecular oxygen and they discussed the importance of nitrate in biological oxidations. But it has been generally accepted that nitrate plays no significant rôle as oxidizing agent in higher animals.

Recently we tried to elucidate the mechanism of nitrate reduction in nature and its physiological meanings. R. Sato and one (Egami) of us (3) have extracted a nitrate reducing enzyme, nitrate reductase from *Escherichia coli* by ultrasonic destruction of the cells and have pointed out that the enzyme is an iron porphyrin protein and probably identical with the cytochrome b of *coli* type with α and β-absorption bands at 560 mμ and 530 mμ respectively. They have also shown that the nitrate—nitrate reductase system is able to act as hydrogen acceptor for the succinate—succinic dehydrogenase system as well as for the reduced form of alloxazine-adenine-dinucleotide. From these facts they (4) have been led to the consideration that the nitrate—nitrate reductase system plays the analogous rôle to that of the Warburg-Keilin-system in the presence of nitrate in place of oxygen.

In order to demonstrate the validity of the consideration, one (Murakami) (5) of us studied the effects of nitrate upon the respiration of resting *Coli.* It may be expected from this consideration, that nitrate in the presence of nitrate reductase may inhibit competitively the oxygen absorption according to the following scheme:



In fact, the inhibition by nitrate has been observed with succinate, formate etc. as substrates, showing that the reactions

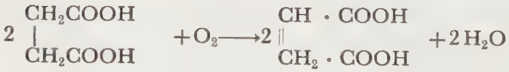


$$\begin{array}{c}COOH\\|\\CH_2\\|\\CH_2\\|\\COOH\end{array} + KNO_3 \longrightarrow \begin{array}{c}COOH\\|\\CH\\\|\\CH\\|\\COOH\end{array} + KNO_2 + H_2O$$

and

$$HCOOH + KNO_3 \longrightarrow CO_2 + KNO_2 + H_2O$$

take place in measurable degree instead of the reactions



$$2\begin{array}{l}CH_2COOH\\|\\CH_2COOH\end{array} + O_2 \longrightarrow 2\begin{array}{l}CH \cdot COOH\\\|\\CH_2 \cdot COOH\end{array} + 2H_2O$$

and

$$2H \cdot COOH + O_2 \longrightarrow 2H_2O + 2CO_2$$

But on the contrary, no inhibition, but an acceleration by nitrate has been observed with glucose, lactate, acetate, and pyruvate as substrates as well as in auto-respiration.

In order to explain this phenomenon, we (5) (6) have been led to the hypothesis that the oxidation of these substrates in the presence of nitrate in aerobiosis proceeds by the way (II) instead of the way (I) in the following scheme:

$$\begin{tikzcd}
\text{Substrate} \arrow[r, dotted, dash] & X \arrow[rr, dotted, dash] \arrow[dr, dash] & & \text{W. K. System} \arrow[r, dash] & O_2 & \text{(I)} \\
 & & Y \arrow[r, dotted, dash] & \text{W. K. System} \arrow[r, dash] & O_2 & \text{(II)}
\end{tikzcd}$$

where X is an intermediate rapidly oxidizable by nitrate—nitrate reductase system to Y, which in turn is rapidly oxidized by W. K. System. The nitrite formed in cource of this reaction may be reoxidized, for example, by the coupled oxidation of Keilin and Hartree as suggested by us (7) in general, and by Heppel and Porter-field (8) in the case of rat liver.

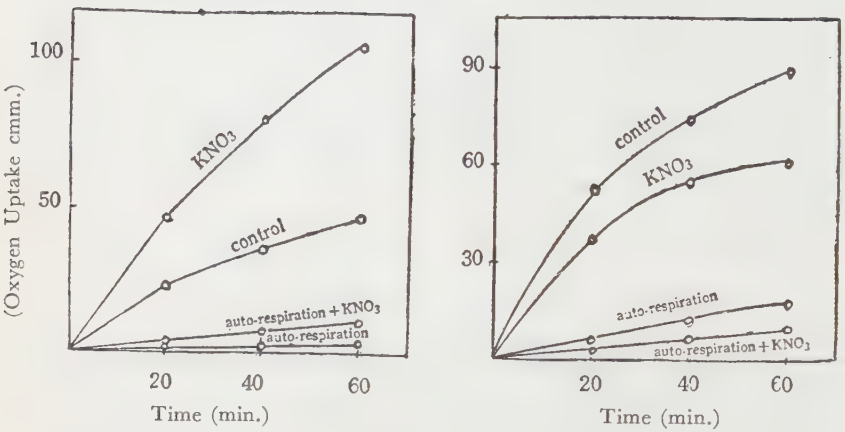
In the present paper some additional experiments on this working hypothesis will be reported.

EXPERIMENTAL

1. Effects of nitrate upon the oxygen absorption of Streptococcus hemolyticus.

The effects of nitrate upon the glucose oxidation by resting *Streptococcus* hemolyticus were studied quite in the same way as with *Escherichia coli* formerly reported. (5) The oxygen absorption was measured by the Warburg's apparatus. The results obtained are comparatively shown with that of *Escherichia coli* in Fig. 1.

Fig. 1



a) Effect of nitrate (KNO_3 $M/450$) in the oxidation of $M/450$ glucose by resting *Escherichia coli* temp, 37°; phosphate buffer $M/45$, pH 7.4.

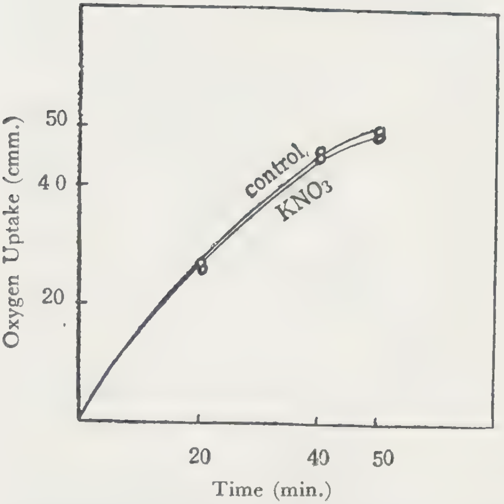
b) Effect of nitrate (KNO_3 $M/450$) on the oxidation of $M/450$ glucose by resting *Strep. hemolyticus*. temp. 37°; phosphate buffer $M/45$, pH 7.4.

By this experiment it is shown that the oxygen absorption in the glucose oxidation by *Streptococcus hemolyticus* is, contrary to that of *Escherichia coli*, not accelerated by nitrate. It may be related to the fact that the former has no nitrate reductase and is unable to reduce nitrate. (9)

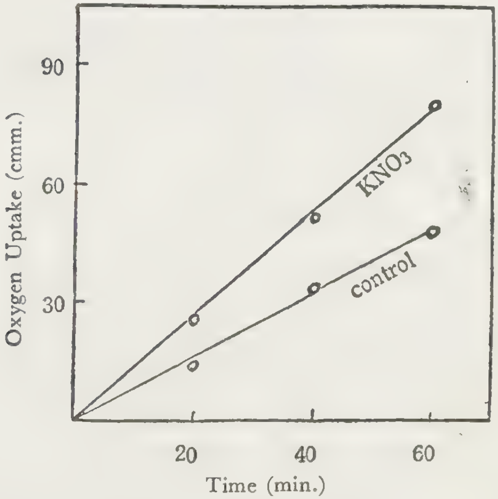
2. Effects of nitrate upon the oxygen absorption of liver and brain homogenates.

The effects of nitrate upon the oxygen absorption in the auto-respiration of mouse liver and mouse brain homogenates as well as upon the acetate oxidation of beef liver homogenates were studied. The results obtained are shown in Fig. 2.

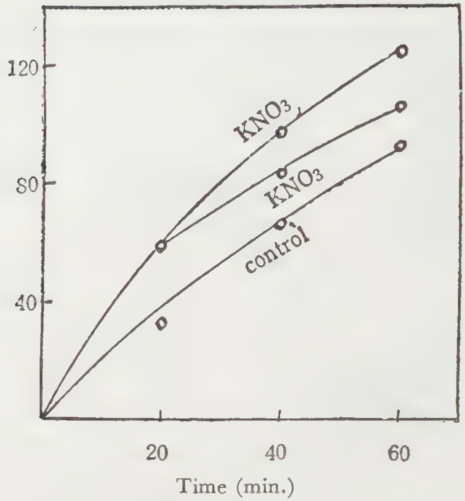
Fig. 2



a) Effect of nitrate (KNO_3 *M*/450) on the oxidation of *M*/450 glucose by mouse brain homogenate. temp. 37° ; phosphate buffer *M*/45, pH 7.2.



b) Effect of nitrate (KNO_3 *M*/450) on the auto-respiration of mouse liver homogenate temp. 37°; phosphate buffer *M*/45, pH 7.2.



c) Effect of nitrate (KNO_3 10^{-2}, $10^{-4}M$) on the oxidation of $M/40$ acetate by beef liver homogenate. temp. 37°; phosphate buffer $M/45$, pH 7.2.

Herei t is shown that the oxygen absorption of liver homogenates is accelerated by nitrate, but that of brain homogenate does not. It may also be related to the fact (10) that only the former has nitrate reductase similar to that of *Escherichia coli.*

Discussion

Although the validity of our hypothesis has not yet been firmly established, it is now quite certain, that the acceleration of oxygen absorption by nitrate in certain cell respirations is due to the existence of nitrate reductase. Moreover it may at least be concluded from the foregoing experiments that the nitrate—nitrate reductase system plays not only the rôle of ultimate hydrogen acceptor, but also the rôle of intermediate oxidation catalysor in certain cell respirations.

As already stated, it has been generally considered that nitrate plays no significant rôle in higher animals. But taking into consideration the existence of nitrate reductase in liver and the remarkable acceleration of its auto-respiration by small quantities of nitrate (10^{-4} *M*), we have been led to consider in contrary to the generally accepted idea, that nitrate might have some important rôle in certain cell respirations in higher animals.

A part of the cost of this study was defrayed from the grant of the Ministry of Education.

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The Journal of Biochemistry, Vol. 37, No. 1

UEBER DIE KETTEN-RING-TAUTOMERIE DER AMINOSÄUREN.

Von

MASAJI TOMITA und YOSHINORI SEIKI

(Received for publication, January 19, 1950)

Auf dem Gebiet der Tautomerie sind der Forschung des letzten Jahrzehntes bedeutende Fortschritte zu danken. Es ist dabei gekennzeichnet durch die Leichtigkeit, mit welcher die beiden Isomeren wechselseitig ineinander übergeführt werden können. Trotz dieser gegenseitigen Umwandlungsfähigkeit gelang es in den meisten Fällen, die beiden Formen durch chemische Merkmale voneinander derart zu unterscheiden, dass die beiden zur Wahl stehenden Strukturformeln mit einem hohen Grad von Wahrscheinlichkeit auf die gefundenen Modifikationen verteilt werden konnten. Neben den chemischen Merkmalen wurden die physikalischen Kennzeichen Gegenstand eifriger Untersuchung. Beide Methoden zugleich werden heute für die Behandlung dieser viel erörterten Probleme benutzt.

Im Laufe der Zeit ist es uns gelungen, namentlich unter Anwendung von röntgenographischen Methoden, die Sonderexistenz der tautomeren Aminosäuren ausser den gewöhnlichen enantiomorphen Verbindungen als solche oder in Gestalt ihrer Derivate im isolierten Zustande, zum Nachweis zu bringen. Derartige Körper sind γ-Amino-β-oxy-buttersäure und Glykokoll.

Diese Verbindungen enthalten ein durch besondere Beweglichkeit innerhalb des Moleküls ausgezeichnetes Wasserstoffatom, welches infolge der Brücken-0-Bindung zwischen C und N, wechselnde Stellung einnimmt. Das charakteristische Unterscheidungsmerkmal dieser Tautomerie ist das, dass unter bestimmten physikalischen Bedingungen eine Form des isomeren Stoffes als labil bekannt ist, wogegon eine andere von isomeren Verbindungen sowohl im festen als such im gelösten Zustande stets als stabil existiert.

Dass nun für die Reaktionsverhältnisse der Aminosäuren neben der gewöhnlichen Kettenform auch die betainartige Ringformel in Betracht kommt, zeigt der Verlauf der Alkylierung, die zu Derivaten beider Formeln führen kann. Behandelt man z. B. γ-Amino-β-oxybuttersäure für sich mit Methyljodid, so gehen drei Methylgruppe bei der ringförmigen Verbindung ausschliesslich an den Stickstoff, wahrend die gewöhnliche kettenförmige Aminosäure unter Eintritt von 2 Methylgruppen an Stickstoff einen Methylester liefert.

Bei der Säurechloridbildung des Glykokolls hat Emil Fischer (1) eine ieteressante Beobachtung gemacht. Nachdem manche Versuche, das gewöhnliche, aus Wasser krystallisierte Gykokoll in das salzsaure Chlorid zu verwandeln, fehlgeschlagen waren, führte eine zufällige Erfindung auf den richtigen Weg. Es ist nun nötig, das Gykokoll in wenig warmem Wasser zu lösen, dann durch einen grossen Ueberschuss von absolutem Alkohol rasch zu fällen, das so erhaltene Präparat sorgfältig bei 100° zu trocknen, zu pulverisieren und durch ein feines Haarsieb zu treiben. Es scheint ihm aber schwierig, den merkwürdigen Unterschied zwischen dem aus Wasser krystallisierten und dem mit Alkohol gefällten Glykokoll richtig zu beurteilen. Er neigte mehr zu der Ansicht, dass es sich um verschiedene Zustände des Glykokolls handelt, die auf Isomerie schliessen lassen. Er wollte jedoch diese interessante Frage nicht engehender behandeln, bis die tatsächlichen Beobachtungen ein vollkommenes Bild geben.

In der voraufgehenden Schilderung der Verhaltens des Glykokolls ist angenommeu, dass in der mit Alkohol gefällten Verbindung eine Hydroxylgruppe vorhanden ist, womit ja auch, deren Fähigkeit, mit Phosphorpentachlorid Säurechlorid zu bilden, in bestem Einklang steht. Das Ausbleiben dieser Säurechloridbildung des sich aus Wasser auskrystallisierten Glykokolls lässt aber noch eine andere Konstitutions-Auffassung naheliegend erscheinen; man kommt so zu der betainartigen Ringformel, die keine Hydroxylgruppe, sondern eine NH_3-Gruppe—also drei direkt an Stickstoff gebundene Wasserstoffatome—aufweist.

Dank einer röntgenographischen Methode von Debye und Scherrerist (2) die Tautomerie von γ-Amino-β-oxy-butteräure klarer zutage getreten. Besonders hat diese physikalische Anwendung sich das grösste Verdienst einer Aufklärung der Glykokoll-struktur zugeschrieben.

Die Glykokoll-isomerie ist wiederholt Gegenstand der Untersuchung gewesen, ohne dass es gelungen wäre, die gesuchten Isomere zu gewinnen. Wir waren deshalb überrascht, dass die Identifizierung beider Tautomere des Glykokolls bloss durch Röntgenographie leicht ausgeführt werden konnte. Durch systematische röntgenographische Untersuchungen wurden die Glykokoll-präparate, die in verschiedenen Fabriken dargestellt worden waren, in ketten- und ringförmige Verbindungen geteilt. Es zeigte sich dabei, dass das nach Sörensen'schem Verfahren (3) synthetisierte Glykokoll ringförmige Struktur hat und das von Kahlbaum bezogene Präparat die dem ringförmigen Glykokoll entsprechende Struktur haben musste.

Sörensen's Verfahren ist eine Malonester-Reaktion, die vom Phthalimido-malonester über das entsprechende Derivat zur Aminosäure führt. Viele Beispiele kennen wir von denselben Reaktionen gelernt, bei denen sich aus Malonesterverbindungen durch intramolekularen Ringschluss heterocyclische Systeme bilden, so z. B. β-Oxycumarin, Tetronsäure und Tetramsäure von Anschütz, (4) α-Pyrrolidincarbonsäure von Willstätter (5) und von Emil Fischer (6) und Bis-α-piperidon β, β-Spiran von Emil Fischer und M. Bergmann (7).

Die Ringbildung bei der Verseifung und Abspaltung von einem Carboxyl aus dem Phthalimido-alkyl-malonester scheint bedingt zu sein durch die Neigung des tertiär gebundenen Kohlenstoffattoms, bei Abgabe des Carboxyls eine ungesättigte Gruppe zu bilden, welche sekundär zur Entstehung des betainartigen Rings Veranlassung geben könnte. In der Tat lässt sich das Hindernis, welches der Entstehung der kettenförmigen Aminosäure hier entgegensteht, leicht dadurch beseitigen, dass man zunachst den Phthälimido-alkyl-malonester durch Verseifung und Abspaltung von einem Carboxyl in die entsprechende

Phthalimido-alkyl-monocarbonsäure überführt. Diese wird nachträglich mit starker Salzsäure gespalten und so entsteht eine kettenförmige Aminosäure.

In den Malonesterreaktionen treffen wir also Belege für die allgemeine Gültigkeit der Erscheinung, dass gerade Ketten von Kohlenstoffatomen besonders leicht durch ein Sauerstoffatom oder ein Stickstoffatom zu einem ringförmigen Komplex geschlossen werden.

Offen geblieben ist nun die Frage, welcher Teilvorgang für das Auftreten der Tautomere der γ-Benzoylamino-β-oxy-buttersäure bzw. der γ-Amino-β-oxy-buttersäure bei der asymmetrischen Synthese mit Hilfe von Brucin verantwörtlich zu machen ist.

Vielleicht wäre der Chinolin-Stickstoff im Brucinmoleküle bei der Verkuppelung mit der Carboxylgruppe der γ-Benzoylamino-β-oxy-buttersäure aus dem drei- in den mehrwertigen Zustand übergegangen. Bei der nachträglichen Abspaltung des Brucins könnte das frei gewordene Carboxyl-Sauerstoff zur Ringbildung Veranlassung geben, infolge der Brücken-O-Bindung zwischen Carboxyl-Kohlenstoff und dem Stickstoff, der aus der Atomgruppe $O{=}\overset{|}{C}{-}\overset{|}{N}{-}H$ durch Tautomerie in Enolform umgewandelt wird.

Im Rahmen unserer fortlaufenden Untersuchungen hat es sich herausgestellt, dass das durch Phthalimidmalonestermethode synthetisierte Glykokoll ringförmige Struktur hat und nicht stabil ist und dass es in der wässerigen Lösung besonders bei einer höheren Temperatur leicht in die Kettenform übergeht. Diese Umwandlung vollzieht sich auch bei der Fällung einer wässerigen Lösung der Ringform in Alkohol. Das chemische Verhalten beider Isomere des Glykokolls ist verschieden. Wie Fischer schon beobachtete, waren Versuche, das ringförmige Glykokoll in das salzsaure Chlorid zu verwandeln, fehlgeschlagen, während eine Anwendung des mit Alkohol gefällten Glykokoll leicht zum Ziel führte. Die von F. Hasse (8) beobachtete Methylenblau-Reduktion des Glykokolls fällt bei dem ringförmigen positiv aus. Bei der Kondensation des kettenförmigen mit Benzaldehyd im Gegenwart von wasserfreiem Natriumacetat wird α-Benzalamino-β-acetoxy-β-phenyl-propionsäure gebildet. Bei Anwendung des kettenförmigen Glykokolls wird diese Verbindung nicht dargestellt.

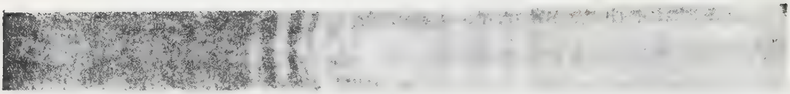
Mit Hilfe der röntgenometrischen Methoden von Debye einerseits und von Laue andererseits wird die Strukturverschiedenheit der beiden Isomere des Glykokolls festgestellt. Nach Dobye erhielt man die den beiden typischen Interferenzbilder (Abbildung I). Die gemessenen Werte der Debye-Ringe stehen, wie man in der folgenden Tabelle ersieht, mit den aus verschiedenen Dimensionen von Laue-, Schwenk- und Drehkristall-Aufnahmen berechneten Zahlen in vollkommener Uebereinstimmung.

TABELLE I

Millers Index hkl	Abstand d Å	Radius der Debye-Ringe (cm)	
		Berechnet	Gemessen
(020)	5.98	1.24	1.24
(011)	4.66	1.60	1.59
(110)	4.41	1.68	1.68
$(1\overline{01})$	4.35	1.71	1.68
(120)	3.71	2.00	2.00
(031)	3.13	2.36	2.41
(130)	3.65	2.45	2.46
(040)	—	2.50	2.55
(140)	2.53	2.97	3.00
(002)	—	2.97	3.00
$(14\overline{1})$	2.47	3.03	3.05
(012)	2.46	3.05	3.05
$(1\overline{22})$	2.45	3.07	3.08
(200)	—	3.18	3.20
(220)	—	3.42	3.35
$(23\overline{1})$	2.14	3.53	3.51
(032)	2.14	3.54	3.51
(141)	2.11	3.59	3.60
$(2\overline{32})$	1.91	3.97	4.00

Abbildung I

Debye-Aufnahme



a. Glykokoll (Kettenformel)

b. Glykokoll (Ringformel)

Schwenk- und Drehkristall-Aufnahme mit Cu-Kα-Strahlung um die 3 Hauptachsen ergaben für die beiden Isomere folgende Dimensionen.

Tabelle II

Struktur des Glykokolls	a	b	c	β	Molekülzahl	Raumgruppe
Kettenform	5.11 Å	12.00 Å	5.43 Å	111° 39′	4	C_{2h}^5
Ringform	5.20 Å	6.17 Å	5.30 Å	115° 00′	2	C_s^2

Wenn man diese Zahlen mit den Ergebnissen von Bernal (9) sowie von Albrecht u. Corey (10) vergleicht, so geht hervor, dass das Glykokoll von den letzten Forschern unserer Kettenformel entspricht und die Zahlen des α-u. β-Glykokolls nach Bernal mit denselben unserer Ketten- und Ringformel übereinstimmen.

Der Vergleich lässt aus Tabbelle III übersehen.

Tabelle III

Untersucher / Dimension	Albrecht u. Corey	Bernal		Tomita u. Seiki	
		α-Glycin	β-Glycin	Ketten-F.	Ringform
a	5.10 Å	5.04 Å	5.18 Å	5.11 Å	5.20 Å
b	11.96 Å	12.10 Å	6.18 Å	12.00 Å	6.17 Å
c	5.45 Å	5.41 Å	5.29 Å	5.43 Å	5.30 Å
β	111° 38′	111° 38′	114° 20′	111° 39′	115° 00′
Molekülzahl	4	4	2	4	2
Raumgruppe		C_{2h}^5	C_s^2	C_{2h}^5	C_s^2

Mehrere Forscher haben schon den isoelektrischen Punkt des Glykokolls bestimmt und dabei wird ein ziemlich grosser pH-Bereich zwischen dem niedrigsten Wert von Bach (11) bei pH 5.8 und dem höchsten Michaelis's (12) bei 6.25 angegeben. Allerdings hat die Mehlzahl der Untersucher (13) ihn in dem Mittelwerte gefunden.

Einen grossen Teil dieser Unterschiede kann man wohl auf Materialverschiedenheiten zurückzuführen, zum Teil vielleicht auch auf die Strukturveränderung der labilen Ringformei in nicht niedriger Temperatur. Um die Frage zu erörtern, welche Bedeutung die Bestimmungsbedingungen für diese Differenzen und damit für den Wert der Messungen überhaupt haben, haben wir den isoelektrischen Punkt des Glykokolls nach der Hiraki'schen (14) Titrationakurven-Methode bei 10° C gemessen und als Versuchsmaterial beide Isomere des Glykokolls herangezogen. Die Resultate sind im folgenden tabellarisch und kurvenmässig dargestellt.

Tabelle IV

pH-Werte der Titrationskurve des Glykokolls

a. Ketten-Form

NaOH	2.5% Glykokoll		5.0% Glykokoll		7.5% Glykokoll	
cc	Millivolt	pH	Millivolt	pH	Millivolt	pH
0.0	426.5	3.015	440.7	3.268	452.8	3.484
2.0	441.3	3.279	459.0	3.594	469.8	3.786
3.0	454.8	3.519	470.7	3.802	482.4	4.011
3.5	465.4	3.708	481.0	3.986	493.8	4.214
4.0	480.4	3.975	497.5	4.280	513.6	4.566
4.2	494.4	4.223	512.4	4.545	527.1	4.807
4.7	688.1	7.674	657.8	7.135	652.8	7.045
4.9	697.3	7.840	677.5	7.486	671.6	7.380
5.3	716.9	8.187	692.4	7.751	689.0	7.690
6.0	732.8	8.470	713.5	8.126	705.5	7.984

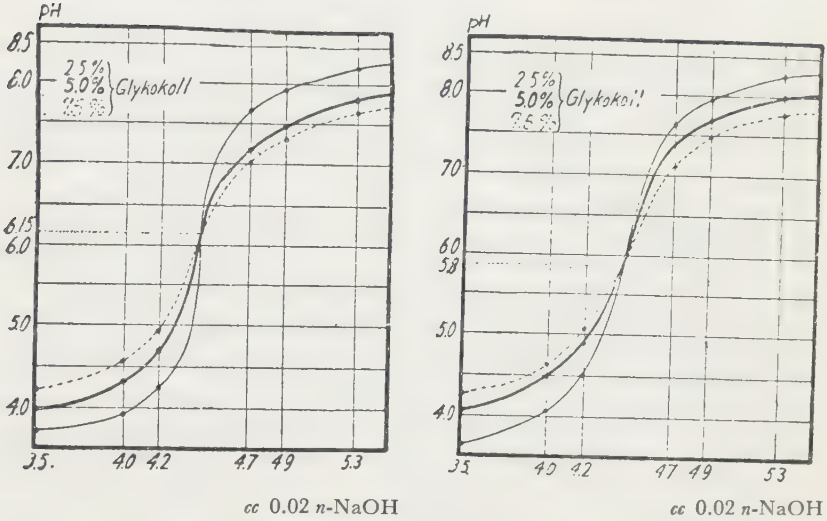
b. Ring-Form

NaOH	2.5% Glykokoll		5.0% Glykokoll		7.5% Glykokoll	
cc	Millivolt	pH	Millivolt	pH	Millivolt	pH
0.0	424.9	2.987	434.0	3.149	451.9	3.467
2.0	442.1	3.275	456.3	3.546	468.2	3.758
3.0	455.5	3.532	472.6	3.836	483.6	4.032
3.5	466.5	3.728	484.5	4.048	495.7	4.251
4.0	488.3	4.116	505.5	4.422	514.2	4.581
4.2	509.0	4.484	529.7	4.851	538.7	5.013
4.7	686.7	7.649	672.5	7.396	666.1	7.282
4.9	702.8	7.936	689.6	7.701	678.1	7.496
5.3	717.5	8.198	702.0	7.922	691.9	7.742
6.0	734.1	8.493	716.8	8.185	709.6	8.059

Abbildung II

Titrationskurve des Glykokolls

a. Ketten-Form *b. Ring-Form*



Isoelektrischer Punkt des kettenförmigen Glykokoils wird also bei pH 6.15 gefunden, während ein pH-Wert von 5.8 bei dem ringförmigen angegebon wird.

Aus unserer heutigen Kenntnis der Tautomerie-Erscheinungen der Aminosäuren ergeben sich für die weitere Forschung zahlreiche Aufgaben. Mit welcher Geschwindigkeit vollzieht sich die Umlagerung, wie wird die Geschwindigkeit durch die Temperatur, durch die verschiedenen Lösungsmittel, durch die chemische Zusammensetzung der Atomgruppen, die mit der labilen Atomgruppe verbunden sind, beeinflusst? Hinsichtiich der biologischen Bedeutung der ringförmigen Aminosäuren ist to gut wie nichts bekannt. Am Eiweissmolokül scheinen es insbesondere die ringförmigen Bausteine zu sein, die die Antikörperbildung und überhaupt die Antigen-Antikörper-reaktionen auslösen. Der Bearbeitung dieser Fragen bieten sich den Experimontatoren noch zahlreiche lohnende Anfgaben. Dass auch unseren Arbeitsrichtungen durch das tiefere Eindringen in die Natur dieser molokülaren Umwandiungen Förderung erwachsen wird, darf man wohl mit Zuversicht hoffen.

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A CONTRIBUTION TO THE METABOLISM OF TRYPTOPHANE

By

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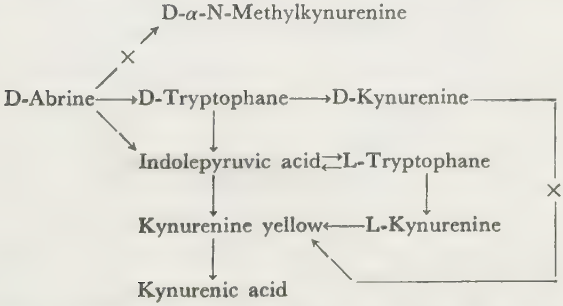
(Received for publication, January 23, 1950)

Though many studies on tryptophane metabolism have been known, Kotake's theory (1931) is decisive on account of his discovery of kynurenine.

It is, however, an open question whether Ellinger-Matsuoka's theory, namely the formation of kynurenic acid from tryptophane by way of indolepyruvic acid, is no more of value. In this connection Komaki's experiments (1944) in our laboratory are worthy of mention.

He administered D-abrine subcutaneously to some rabbits, and obtained a relatively large amount of kynurenic acid and unchanged matter together with a small quantity of kynurenine yellow-like-substance, but no kynurenine.

The possible ways to explain such metabolism are as follows:



D-tryptophane and indolepyruvic acid may give us a key to solve the problem.

The present paper describes the experiments carried out to elucidate the way of the formation of kynurenic acid from these two substances.

EXPERIMENTAL

1. Examination of D-tryptophane in the animal body.

I) D-Tryptophane, which was prepared from L-tryptophane according to Majima (1936), m. p. 244°–246°. Its rotation was+32.34°.

II) Procedure of the urine analysis after tryptophane administration:

Male rabbits were kept in metabolism cages and given polished rice *ad libitum.*

Tryptophane was administered subcutaneously twice or thrice in a suitably divided amount. The 48 hour urine was collected, 25% H_2SO_4 was added to make its concentration 5%. It was kept in the ice-box, laying ether on it.

After the precipitate (crude kynurenic acid A) was centrifuged off, 10% $HgSO_4$ solution (in 5 vol.-% H_2SO_4) was added and the precipitated mercuric double salt was filtered off after 24 hours.

The residue, suspending in water, was decomposed with H_2S and the HgS resulting was filtered off. The H_2SO_4 was neutralized with $Ba(OH)_2$ to as extent being slightly acidic to congo red, the filtrate from $BaSO_4$ was evaporated *in vacuo* and kept in the ice-box. As a precipitate appeared, it was filtered off (crude kynurenic acid B). The crude kynurenic acid A and B were dissolved altogether in diluted ammonia water and HCl was added, the precipitate was filtered and recrystallized from a large amount of boiling water.

The mother liquor of the crude kynurenic acid B, removing the excess of H_2SO_4 repeatedly, was evaporated *in vacuo* under faint acidity. Special caution was taken in that procedure not to alkalinify the reaction medium.

If the expected sulfate of "kynurenine body" precipitates, it is to be filtered off, and removing the residual H_2SO_4 quantitatively the filtrate is to be evaporated *in vacuo* to obtaine the unchanged matter.

III) Results:

The results of analysis were recorded in Table I.

Table I

Rabbit No. Body Weight	Administered Amount	Kynurenic Acid	Kynurenine Body	Residual Fraction
No. 1 1850 g. ♂	L-tryptophane 3.0 g.	123 g. Jaffe test (+) Kretschy test (+) Bariumsalt formation (+)	0.38 g. Otani-Honda test (+) Ninhydrine test (+)	Slight Hopkins-Cole test (−)
	D-tryptophane 1.0 g.	None	Slight* Otani-Honda test (−) Ninhydrine test (−)	Slight Hopkins-Cole test (−)
No. 2 1850 g. ♂	D-tryptophane 0.7 g.	None	Slight* Otani-Honda test (−) Ninhydrine test (−)	Slight Hopkins-Cole test (−)
	D-tryptophane 0.66 g.	None	Slight* Otani-Honda test (−) Ninhydrine test (−)	Slight Hopkins-Cole test (−)

* As Otani-Honda's reagent was added, they showed brown color but this color was moved absolutely into butanole-layer.

2. *Examination of indolepyruvic acid in the animal body.*

I) Preparation of indolepyruvic acid:

Indolealdehyde was prepared according to Majima-Kotake

(1922) and indolepyruvic acid to Ellinger-Matsuoka (1920) subsequently. The latter was white prisma, decomposed at 210°C.

$C_{11}H_9NO_3$	Calculated	N	6.90
	Found	N	7.03

II) Administration and the urine analysis. Indolepyruvic acid, dissolved in warm water and neutralized with sodium bicarbonate, was injected subcutaneously in the rabbit. The 48 hour urine was collected and analysed as above mentioned.

The harmfull by-effects recorded by Ellinger-Matsuoka were not observed.

III) Results:

They were recorded in Table II.

TABLE II

Rabbit No. Body Weight	Administered Amount of Indolepyruvic Acid	Kynurenic Acid	Kynurenine Body	Tests of Residual Fraction
No. 1 3000 g. ♂	0.5 g.	Slight in the (B) part	Slight* Otani-Honda test (+)	Hopkins-Cole test (−) Bromine test (−)
No. 2 2800 g. ♂	2.0 g.	0.170 g. Kretschy test (+)	Slight* Otani-Honda test (+)	Hopkins-Cole test (−) Bromine test (−)
No. 3 3000 g. ♂	2.0 g.	0.248 g. Kretschy test (+)	Slight* Otani-Honda test (+)	Hopkins-Cole test (−) Bromine test (−)

* Every kynurenine body showed a positive Otani-Honda test. (violet color was not extracted by butanole).

The kynurenine bodies were collected altogether to crystallize. But, in spite of all efforts, no typical crystal of kynurenine sulfate could be obtained.

This slightly green yellow residue showed a positive Otani-Honda test and Pauli's diazo reaction, but no ninhydrin test*. After a month, it discolored to brown and faintly responded positively to the Kretschy color test.

* By the method of paperchromatography (the solvent: collidine 2, lutidine 5, saturated with water) the Rf-value of the kynurenine isolated from the urine after L-tryptophane administration was 0.28 (alanine: 0.25, leucine: 0.53 as control). But this residual fraction whereas showed no ninhydrin spot.)

These particularities do not correspond to kynurenine, but to "kynurenine yellow".

Discussion

Berg (1934) showed that both D-and L-tryptophane are epually effective in supporting growth in the rat. On the other hand, the apparent excretion of kynurenic acid from the rabbit was so slight by the administration of D-tryptophane (either *per os* or subcutaneously) as to indicate no appreciable production from this modification.

Nagao (1937) determined the iodic acid keto-value to judge the rise and fall of ketonic acids in the urine, and found D-tryptophane gave higher keto-values in the cases of subcutaneous injection to rabbits and dogs than of administration *per os*. But kynurenine bodies were formed more easily *per os* than injection. (Yahito Kotake have also obtained D-kynurenine after the *per os* application of D-tryptophane to the rabbit).

According to Nagao, the excretion of unchanged D-tryptophane was demonstrated by means of the bromine test, and no kynurenic acid was obtained in both cases of injection and *per os* administration.

On indolepyruvic acid, we have the welknown study of Ellinger-Matsuoka (1920). They found a relatively much smaller conversion (1.2–11.7% of the administered amount) of this derivative, than of L-tryptophane, into kynurenic acid.

Some other investigators have also obtained comparatively slight kynurenic acid with the ketonic acid (4.3–7.4% by Bauguess and Berg (1934), 3.3–17.4% by Ootani *et al.* (1939)). So Kotake claims that indolepyruvic acid is by no means the direct producer of kynurenic acid, but is converted to L-tryptophane in the animal body and then through L-kynurenine finally to kynurenic acid.

From the present study in Table I and II, it may be concluded as follws:

1) Kynurenic acid, kynurenine body and the unchanged matter were not recovered in the rabbit urines at all after the subcutaneous administration of D-tryptophane.
2) Kynurenic acid corresponding to 8.5-12.9% of the administered amount was obtained in the rabbit urines after the subcutaneous injection of indolepyruvic acid, but no definite kynurenine was found. A small amount of amorphous substance in kynurenine fraction gave a positive Otani-Honda test and every effort was made to isolate crystalline kynurenine sulfate from it, but in vain. Furthermore we could not confirm the existence of kynurenine in it by paperchromatography. It is evident, therefore, that no kynurenine was produced in spite of the amount of kynurenic acid. The substance, which showed Otani-Honda's reaction, might be kynurenine yellow. No other matters were obtained.

Now, we are very interested in Komaki's report that a relatively large amount of kynurenic acid and a small amount of kynurenine yellow-like-substance were obtained together with unchanged matter by the administration of D-abrine.

It is clear that D-abrine does not turn into D-tryptophane and that no kynurenine will occur from it but kynurenine yellow. It is quite similar to the case of indolepyruvic acid of my work. Namely, it may be also indicated that D-abrine and indolepyruvic acid will be directly, converted into kynurenic acid through kynurenine yellow, but not through kynurenine.

From this standpoint of view, Ellinger-Matsuoka's way is to consider to be worthy of existence as the secondary course of tryptophane metabolism together with Kotake's primary or principal way.

SUMMARY

Our observations on the metabolism of D-tryptophane and indolepyruvic acid are in general agreement with those of other investigators.

After the subcutaneous administration of indolepyruvic acid to rabbits, the output of kynurenine was not to ascertain, not only by isolation of crystalline substance, but also by paper chromatography, although the kynurenine yellow-like substance was obtained.

It is very probable that indolepyruvic acid will be converted directly to kynurenic acid, not through kynurenine anyhow. Ellinger-Matsuoka's way is, therefore, applicable as the secondary way to form kynurenic acid from tryptophane.

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ON THE FATE OF SQUALENE IN THE ANIMAL BODY

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Squalene, or spinacene was isolated from the liver-oil of some Squalidae by Tsujimoto (1906, 1914) and independently by Chapmann (1917). Its constitutional formula was studied by Kubota (1918, 1922), Chapmann (1923), Heilbron (1926, 1929, 1930) and Karrer (1930). Its fate in the animal body was discussed repeatedly as a source of cholesterol by Channon (1926, 1937), Heilbron (1926), Minovici (1933), Robinson (1934), and Vanghelovici (1937), and denied by Channon and Robinson. I was much interested in its oxidation *in vivo*, as Hirohata, other colleagues and I were concerned in the investigation of the ω-oxidation of rhodinic acid (1947). The rhodinic acid was pointed out as a remedy of tuberculosis by Katsura *et al.* (1941–44), and oxidized to dihydro-Hildebrandtic acid in the animal body. Squalene resembles rhodinic acid in respect to the constitution, viz. side chains and double bonds.

The following article shows that in rabbits squalene will be partly decomposed and oxidized at the double bonds and produces succinic and laevulinic acids, and a small part of it will be stored a relatively long time in the liver. It is also assumed that the squalene will be eliminated by the kidney and the digestive tract.

EXPERIMENTAL

Expt. A. 2.5 ml. squalene in emulsion was administered subcutaneously 4 times in 2 days to each of 3 rabbits weighing about 2–2.1 kg. respectively. Their urine were collected for a week afterwards (in total 6,019 ml.), acidified faintly with H_2SO_4 and extracted with ether for 72 hours. From the ether extract were obtained benzoic and hippuric acids in the acidic fraction and about 0.5 ml. of an oily substance in the neutral fraction. The latter, saturated with dried gas of HCl, turned into crystalline substance, which melted at 107°~125°.

Analysis: 5.22 mg. substance 6.78 mg. AgCl
$C_{30}H_{56}Cl_6$ calculated Cl 33.81
found ,, 32.13

Expt. B. 10 ml. spualene in emulsion injected subcutaneously during 6 days divided into 12 times to each of 3 rabbits weighing 1.6~1.8 kg. Their urines were collected for several weeks (19,130 ml. in toto) and extracted with ether. About 60 mg. crystal in prism was isolated from the acid fraction of the extract. It was soluble in water melted at 183°~4°, showed no depression of melting point mixed with succinic acid.

Analysis: 3.998 mg. substance 1.851 mg. H_2O 6.030 mg. CO_2
4.106 ,, ,, 1.836 ,, ,, 6.118 ,, ,,
4.239 ,, ,, 1.913 ,, ,, 6.351 ,, ,,
$C_4H_6O_4$ calculated C 40.68......................H 5.12
found C 41.17, 40.66, 40.89.: H 5.18, 5.00, 5.05

Titration: 3.94 mg. substance 3.32 ml. N/50 NaOH (alcoholic)
$C_4H_6O_4$ calculated Mol. weight 118.09
found ,, ,, 118.70

Micro-Rast: 7.69 mg. substance 108.91 mg. campher $\triangle$23.3
$C_4H_6O_4$ calculated Mol. weight 118.09
found ,, ,, 121.18

A small amount of benzoic and hippuric acids were also obtained from the acid fraction. About 0.5 ml. oily substance was isolated from the neutral fraction of the extract, which turned crystalline squalene hexahydrochloride by saturation with dried HCl gas, m. p. 116°~125°.

Analysis: 23.41 mg. substance 31.07 mg. AgCl
$C_{30}H_{56}Cl_6$ calculated Cl 33.81
found ,, 32.83

Expt. C. 10 ml. squalene in emulsion was administered to both two rabbits (2.1, 2.4 kg.) subcutaneously divided into 24 portions during 12 days. Their urines were collected for 50 days (23,391 ml. in toto) made alkaline faintly and concentrated on a water bath, acidified slightly with dilute H_2SO_4 and extracted with ether. A small amount of unchanged squalene was obtained from its neutral fraction. Its hexahydrochloride was prepared as usual, m. p. 117°～125°.

Analysis: 9.47 mg. substance 12.87 mg. AgCl
$C_{30}H_{56}Cl_6$ Calculated Cl 33.81
found ,, 33.64

With a considerable amount of hippuric and benzoic acids, other acidic substances were obtained, easily soluble in water, which were crystallized slowly after keeping it a long time in a dessicator in a cold place, melted at 28.5°～30.0°, boiled at 24.5°, was very hygroscopic and showed no depression of the melting point mixed with laevulinic acid.

Analysis: 3.940 mg. substance 7.475 mg. CO_2 2.470 mg. H_2O
$C_5H_8O_3$ calculated C 51.70 H 6.95
found ,, 51.74 ,, 7.01

Titration: 4.08 mg. substance 3.39 ml. N/100 NaOH
5.45 ,, ,, 4.51 ,, ,,
$C_5H_8O_3$ calc. Mol. weight 116.11
found ,, 119.97, 121.41

Micro-Rast: 6.01 mg. substance 106.11 mg. campher $\triangle$18.95°
$C_5H_8O_3$ calculated Mol. weight 116.11
found ,, 119.5

The Ba salt of this acid was resinous and easily soluble in water. The Ag salt was plate-like crystal and easily soluble in water, too.

Analysis: 7.97 mg. Ba-salt 4.97 mg. $BaSO_4$
$(C_5H_7O_3)_2Ba$ calculated Ba 37.38
found ,, 36.68
9.41 mg. Ag-salt 6.01 mg. AgI
$C_5H_7O_3Ag$ calculated Ag 48.63
found ,, 48.39

p-Bromphenacylester of it was prepared in the usual way, m. p. 83.5°~84.0°.

Analysis:	4.82 mg. substance	2.88 mg.	AgBr
	$C_{13}H_{13}O_4Br$	calculated	Br 25.43
		found	,, 25.53

The phenylhydrazone of it melted at 104°~105°.

Tetranitromethane reaction was negative.

Expt. D. As the output of the oxidation products and unchanged squalene is very little, it is most probable that it will be stored in some tissues. The quantities of squalene in each tissue or organ were estimated as follows: Rabbits were killed after 4 hours or 90 days of the last administration of squalene. Each tissue or organ was weighed, and liquidified by heating with conc. NaOH, evaporated to a syrup and extracted with alcohol and ether successively. The ether was distilled off from the extract, saponified with alcoholic KOH. The alcohol was removed and extracted with ether, the ether extract dried with Na_2SO_4 and evaporated. Hexahydrochloride was prepared as usual and weighed. The following table shows the results.

Storage of Squalene in Organs aud Tissues

	Killed on 90th day after the last injection		Killed at 4th hour after the last injection	
	Weight g.	Sq. 6HCl mg.	Weight g.	Sq. 6HCl mg.
Liver	83	7.11	69	14.31
Muscle	68.1	11.50	550	17.14
Skin			235	12.42
Stomach & Intestines	217	4.8	378	6.22
Lung	10	0	12	0.51
Kidney	20	0	19	0
Brain	13	0	8	0
Heart	5	0	10	0

N. B. It is very probable that the high content of squalene in muscle and skin is due to its residue remained without being absorbed.

DISCUSSION

According to B. Kubota (1918, 1922), squalene is oxidized in succinic and laevulinic acid by ozone. Similar substances were obtained in the urine. The amount of them, however, is very small; it should be attributed, for one thing, to a difficulty of its oxidation *in vivo*, in spite of many double bonds and side chains, for another, to further oxidation or speedy concealment in some tissues or organs. Elimination of unchanged squalene from the kidney and digestive tract for a long time and long storage in the liver will be considered as the other evidences of difficulty of oxidation *in vivo*. The short hold-up of squalene in the lungs is worthy of remark, as the absorption must not have taken place through the thoracic duct. There are very few reports concerning the fate of aliphatic hydrocarbons in the animal body. It might be concluded from our experiments that they are rather difficult to oxidize *in vivo*. It seems that, especially, the methyl group of aliphatic hydrocarbons is hardly oxidized. Moreover it is conceivable that the diffusion of spualene through the animal cells is very slow. It is of great interest that squalene is stored a long time not only in the liver of the shark but also of higher mammals.

SUMMARY

1. In the body of the rabbits, squalene will be oxidized in succinic and laevulinic acids to a small extent.

2. A small portion of the administered squalene will be eliminated from the kidney and digestive tracts, and another small part will be stored principally in the liver longer than 3 months.

3. It is assumed that the oxidation of squalene in the animal body is not easy and it might be common to other aliphatic hydrocarbons.

4. The diffusion of squalene through the animal cells seems very slow.

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ON THE SPECIFICITY OF FICIN

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Knowledge is rather meager regarding the specificity of ficin action, a proteolytic enzyme system of fig sap. The present investigation is concerned with the behaviors of substrates, genuine and synthetic, toward the ficin.

It is briefly described here about the studies of vermicidal effect of sap filtrate on human *Ascaris.*

Results and Considerations

The data of the optimum pH available are considerably different for substrates: pH 7 for fibrin (at 50°) (1), pH 6.5 for coagulum of milk (2), pH 6 for benzoyldiglycine (3), and pH 5 for gelatin (4). In the present experiments I have found that for the hydrolysis of gelatin and casein the active optimum range extends from pH 4 to 5 (pH 4.5 as optimal point) (Table I). The rate of the hydrolysis of different proteins decreases in the order: edestin, gelatin, casein, egg albumin, and ricin (Table II). It is notable that edestin is easily digestible, but on the contrary Kazama (3) reported the resistance for the ficin action of the plant protein such as edestin.

Peptones have the same optimum pH range as genuine proteins. The results suggest the presence in ficin of a cathopeptonase, known in plant papainase as well as in tissue protease (Table III).

It was also confirmed that, like the intracellular enzymes such as papain and bromelin, ficin can split dipeptides such as diglycine, glycyl-L-leucine and DL-leucylglycine at neither acid nor alkaline reaction. This is in agreement with the findings of Kazama who was able to find no spliting of diglycine and DL-leucylglycine (Table IV).

But it should be here pointed out that of the tripeptides studied both DL-leucyldiglycine and DL-leucylglycyl-DL-phenylalanine (β) were markedly split by ficin at pH 4.5, from the enzymatic hydrolyzate of the former leucine being isolated (Table IV). It is evident therefore that DL-leucyldiglycine was split into leucine and diglycine. Willstätter (5) reported that a tripeptide leucylglycylleucine was broken down into a dipeptide and an amino acid by papain-HCN, which Bergmann (6) denied. He concludes that tripeptide-spliting action does not exist in plant papainase. In view of ficin specificity it should be assumed that, contrary to tryptic aminopeptidase which widely distributes in animals and plants, a catheptic aminopeptidase may be responsible for the hydrolysis of DL-leucyldiglycine at pH 4.5. From this observation of the hydrolysis point of DL-leucyldiglycine, it is apparent that DL-leucylglycyl-DL-phenylalanine (β) is also split into leucine and glycylphenylalanine (β).

Hippuric acid, acetylglycine, acetyl-L-glutamic acid, chloroacetyl-L-leucine and chloroacetyl-L-phenylalanine were not broken down by the enzyme, as indicating therefore no presence of hippuricase and acylase action (Table IV).

It is interesting to note, however, that benzoyl-DL-leucylglycine and benzoyldiglycine were remarkably split at pH 4.5 and that from the enzymatic split-product of the latter there was directly isolated hippuric acid (Table IV). The site of hydrolysis was therefore determined by the terminal carboxyl group ; this finding should be attributed to a catheptic carboxypeptidase action of ficin.

It is also ascertained that the sap filtrate shows a strong anthelmintic effect of human *Ascaris*, dissolving the worm body.

EXPERIMENTAL

Enzyme Preparation—The freshly collected milky sap from unripe fig (*Ficus carica*) has the pH of 5.6. The crude sap was filtered twice by suction (sap filtrate). One volume of the sap filtrate was precipitated with 3 volumes of acetone. The treatment with water and acetone was repeated by redissolving the acetone precipitate in the original volume of water and reprecipitating it with 3 volumes of acetone, and the acetone powder finally obtained (yield, 10–11 per cent) was kept in a vacuum desiccator over P_2O_5 (acetone powder). Throughout the course of this work, the sap filtrate or an equivalent amount of the acetone powder, ficin (7), calculated as 10 per cent, were tested as the source of enzyme.

Test Mixture—Each test mixture consists of enzyme and substrate solution. The control contains only enzyme solution without substrate. These test solutions were incubated at 37° under toluene. The extent of hydrolysis was followed by titrating the liberated carboxyl groups with 0.1 *N* NaOH per 4 ml. aliquot of test solutions according to the formo method of Sörensen. The difference of titration values of main and control solutions were given as acidity increase in the tables.

Table I

Optimum pH of Ficin Action

The following test mixtures were adjusted to their pH with dilute HCl or NaOH and kept at 37° under toluene. The increase of acidity was given in ml. of 0.1 *N* NaOH per 4 ml. of test solution.

Acidity increase in 4 ml. digestate (ml. 0.1 *N* NaOH)				
Substrate	Gelatin		Casein	
Test mixture	40 ml. of 1% solution+0.4 ml. of sap filtrate		25 ml. of 4% solution+0.3 ml. of sap filtrate	
	Time (hrs.)		Time (hrs.)	
pH	5	24	24	48
2	0.10	0.28	—	—
2.5	—	—	0.38	0.52
3	0.11	0.31	—	—
4	0.24	0.37	1.37	1.82
5	**0.42**	**0.56**	**1.55**	**1.96**
6	0.13	0.14	0.68	0.86
7	−0.04	0.15	0.43	0.44
8	0.01	0.06	0.44	0.42
9	0.05	0.08	0.40	0.41

TABLE II

Hydrolysis of Genuine Proteins by Ficin at pH 4.5

Test mixture: 20 ml. ot 4 per cent substrate solution + 0.2 ml. of sap filtrate, adjusted to pH 4.5 with dilute HCl; incubation at 37° under toluene.

Substrate \ Time (hrs.)	5	24	72
Edestin*	1.69	3.51	4.61
Gelatin	1.29	2.25	3.15
Casein	1.18	2.31	3.13
Egg albumin (E. Merck)	0.49	1.32	2.03
Ricin	0.07	0.15	0.18

Acidity increase in 4 ml. digestate (ml. 0.1 *N* NaOH)

* Dissolved in 10 per cent NaCl solution.

TABLE II

Hydrolysis of Peptone from Different Sources by Ficin at pH 4.5 and 7.5

Test mixture: 20 ml. of 2 per cent peptone solution + 0.2 ml. of sap fitrate, adjusted to their pH with dilute HCl or NaOH; kept at 37° under toluene.

Acidity increase in 4 ml. digestate (ml. 0.1 *N* NaOH)

Substrate \ pH	4.5			7.5		
	Time (hrs.)			Time (hrs.)		
	24	72	120	24	72	120
Polypeptone (Ta)	0.21	0.33	0.38	0.08	0.01	0.07
Peptone (Te)	0.23	0.41	0.61	0.03	0.15	0.15
,, (Ue)	0.13	0.30	0.47	0.04	0.01	0.00
,, (Witte)	0.43	0.50	0.67	0.10	0.13	0.12*

* Digested at pH 7.0.

Table IV

Hydrolysis of Synthetic Peptides by Ficin

Test mixture: 20 ml. of peptide solutions of a certain concentration + 0.2 ml. of sap filtrate, adjusted to their pH with dilute HCl or NaOH; kept at 37° under toluene.

Substrate	Final concentration (moles)	pH	Acidity increase in 4 ml. digestate (ml. 0.1 *N* NaOH)		Isolated products
			24 hrs.	72 hrs.	
Diglycine	0.05	4.5	0.02	0.01	
		7.8	0.03	0.00	
Glycyl-L-leucine	0.025	4.5	0.00	0.00	
		7.8	−0.02	−0.02	
DL-Leucylglycine	0.025	4.5	0.00	0.02	
		7.8	0.01	0.02	
Triglycine	0.05	4.5	0.01	0.01	
		7.5	0.02	0.03	
DL-Dileucylglycine	0.05	4.5	0.05	0.12	
DL-Leucyldiglycine	0.05	4.5	**0.34**	**0.78** (39% splitting)	Leucine
DL-Leucylglycyl-DL-phenylalanine (β)	0.05	4.5	**0.34**	**0.43**	
Hippuric acid	0.05	5	0.00	0.02	
		7.8	−0.01	−0.01	
Acetylglycine	0.05	5	0.00	0.02	
		7.8	0.02	0.01	
Acetyl-L-glutamic acid	0.025	5	0.03	0.02	
		7.8	0.01	0.00	
Chloroacetyl-L-leucine	0.2	5	−0.03	−0.01	
Chloroacetyl-L-phenylalanine	0.05	5	−0.03	0.01	
Benzoyldiglycine	0.05	5	**1.15**	**1.58** (82% splitting)	Hippuric acid (67% theory)
		7.8	0.23	0.23	
Benzoyl-DL-leucylglycine	0.05	5	**0.17**	**0.32**	

Isolation of Leucine from DL-*Leucyldiglycine Digestate*—A mixture of 20 ml. of 0.05 *M* DL-leucyldiglycine and 0.2 ml. of sap filtrate was adjusted to pH 4.5 with dilute hydrochloric acid and kept at 37° under toluene. After 3 days (39 per cent splitting), the reaction mixture was neutralized with NaOH and filtered. The filtrate was then concentrated under reduced pressure, from which was obtained a sparingly soluble crystalline product. It was recrystallized from 80 per cent alcohol in characteristic spherical balls having a radial structure, and when heated gently in an open tube it sublimed and emitted a characteristic odor of isoamylamine.

Isolation of Hippuric Acid from Benzoyldiglycine Digestate—0.59 g. of benzoyldiglycine, 2.5 ml. of *N* NaOH and 3 ml. of sap filtrate were made up to 100 ml. with 0.1 *M* citrate buffer (pH 5) and kept at 37° under toluene. After 4 days (100 per cent splitting), the reaction mixture was acidified to Congo red with concentrated hydrochloric acid and extracted with ether in a continuous extractor for 12 hours. By evaporating the ethereal solution a yield of 0.30 g. of recrystallized material was obtained, or 67 per cent of the theory. It melted at 188°-189°. The mixed melting point with an authentic sample of hippuric acid showed no depression.

Anthelmintic Ability of Ficin—The anthelmintic properties of ficin (sap filtrate) have been investigated *in vitro* on human *Ascaris* to determine the action of ficin on the live worms. The action of pepsin and pancreatin was also examined.

As shown by Robbins (7), 0.5 per cent solution of sap filtrate in physiological saline solution caused at pH 8 and 37° the death of the worms in 5 hours and dissolved them completely in 24 hours.

While in 3 per cent saline solution of pepsin (commercial) at pH 2 and 37° were dead all the worms in 48 hours, showing several lesions on the body, they were living over 48 hours at pH 8 and 37° in 3 per cent saline solution of pancreatin (commercial).

The worms were also dead in 24 hours at pH 5 or 8 and 37° in 0.1 or 0.3 per cent saline solution of sap filtrate incubated previously with pepsin (5 per cent) at pH 2 and 37° for one hour. In 5 per cent saline solution of pepsin only they were living at pH 5 and 37° for 24 hours. It is evident therefore that the vermicidal ability of ficin can escape completely digestion by pepsin.

The worms were living at pH 5.8 and 37° over 4 days in 1 per cent saline solution of sap filtrate which is heated in boiling water for 5 minutes, while in the saline solution of the fresh sap filtrate they were dead in 2 to 3 hours and went completely into solution in 19 hours.

SUMMARY

1. The pH of optimal activity for gelatin, casein and peptone hydrolysis by ficin is from 4 to 5.
2. Dipeptides are not split by ficin.
3. In the enzymatic hydrolysis of DL-leucyldiglycine at pH 4.5 the isolation of leucine suggests a catheptic aminopeptidase action of ficin.
4. Likewise, the isolation of hippuric acid from benzoyldiglycine digestate leads to the assumption of the presence of a catheptic carboxypeptidase in ficin.
5. In the action of ficin there is no indication of hippuricase and acylase action.
6. The fig sap and also pepsin-treated sap have the vermicidal effect dissolving the worm body, while the boiled sap or pancreatin has no such effect. The anthelmintic ability of ficin is resistant against the digestion by pepsin and pancreatin (8), but it is inactivated by heating in boiling water for 5 minutes.

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CHEMICAL DETERMINATION OF VITAMIN D

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The chemical determination of vitamin D in pure solution or high concentrate is not difficult. The methods, which have been proposed, depend upon the following principles. (I) Estimation of the extinction coefficient at 265 mμ (Fuchs). (1) (II) Colorimetric estimation of the purplish red color, produced by the reaction with aluminium chloride (Halden, (2) Tzoni). (3) (III) Colorimetric estimation of the yellowish orange color produced by the reaction with antimony trichloride (Brockmann *et al*, (4) Emmerie *et al*, (5) Woıff, (6) Ritzert, (7) Milas *et al*, (8) Nield *et al*). (9) (IV) Colorimetric estimation of the color produced by the reaction with activated glycerol dichlorohydrin containing acetyl chloride or other acid halide. (Sobel *et al*, (10) Campbell (11)).

However the estimation of vitamin D in fish oil or liver oil is usually not possible owing to the presence of interfering substances. The separation of vitamin A and D with "Montanerde" is according to Emmerie *et al* (5) only applicable to the concentrate, containing more than 48,000 I. U. of vitamin D per ml. The complete removal of carotenoids with maleic anhydride, as is aeported by Milas (8) was not confirmed by our experiments, in which steroids and carotenoids were not completely removed by the proposed procedure, while at the same time vitamin D was partially destroyed. Having studied the fundamental conditions of color reaction, chromatographic fractionation of vitamin A and D and the effect of the treatment of maleic anhydride upon carotenoids and steroids, we succeeded in developing the procedure for the estimation of vitamin D in liver oil.

PRINCIPLE

The nonsaponifiable fraction of liver oil is chromatographically separated into vitamin A and D fractions. The latter being divided into two equal parts is placed in each test-tube, one of which is treated with maleic anhydride at 80°C for 10 minutes, whereby more than 50% of the existing carotenoids and steroids are colorimetrically destroyed, while vitamin D remains unchanged. The other tube is treated with maleic anhydride at 100°C for 30 minutes, whereby vitamin D is destroyed completely but the steroids and carotenoids remain the same as after treatment at 80°C for 10 minutes. The antimony reagent is added to each tube and the developed color is estimated photometrically. From the difference of extinctions of both treatments the content of vitamin D is calculated.

REAGENTS

1. Methanol.
2. Benzene.
3. 5 % of KOH in ethyl alcohol.
4. 5 % of KOH in 60 volume % of methanol.
5. Activated alumina "BL6" of the Nippon Aluminium Company, Setagaya, Tokyo.

This, corresponds to about the second degree of Brockmann's preparation, with the average particle size of 50–70μ and pH about 6.

6. Petroleum benzine free from peroxide.
7. Petroleum ether free from peroxide.
8. Acetone free from peroxide.
9. Dioxane free from peroxide.
10. 10 % solution of maleic anhydride in dioxane.
11. 0.5 *N* KOH in ethyl alcohol.
12. Chloroform.

Chloroform is washed with an equal volume of water three times and dehydrated with anhydrous potassium carbonate. After standing in dark cool place overnight, the supernatant can be used without further distillation.

13. Antimony trichloride.

Pure preparation is dehydrated in desiccator.

14. Acetic anhydride.

15. Antimony reagent.

To 20 ml. of 0.2 g. per ml. solution of antimony trichloride in chloroform is added 10 drops of acetic anhydride. The exact concentration of the antimony solution being estimated in the following way, the necessary quantity of chloroform is added to make exactly 0.2 g./ml. solution.

1 ml. of the solution under test is mixed with 20 ml. of 0.1 g./ml. potassium sodium tartarate solution. 2 g. of sodium bicarbonate is added and the mixed solution is titrated with $N/10$ iodine solution, starch being used as an indicator. 1 ml. of iodine solution corresponds to 11.4 mg. of $SbCl_3$.

16. Hydrogen gas.

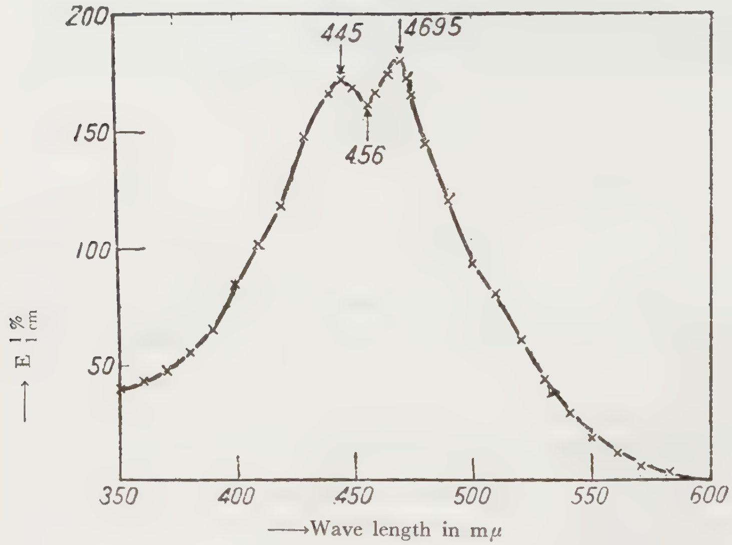
17. Carbonic anhydride gas.

18. X pigment.

This is the pigment, which behaves chromatographically just in the same way as vitamin A and makes the position of the vitamin visible.

It is prepared from red pepper in the following way. About 10 g. of red pepper is cut into small pieces and ground with a small quantity of quartz sand, extracted with metanol and benzene and saponified. The benzene layer being evaporated, the residue is dissolved in petroleum benzine, and chromatographically developed, as is described below in the procedure. Carotenes come out almost completely, when 15 ml. of 10 % acetone-petroleum benzine solution has passed through the column. After about 30 ml. of the developing solution having flowed out, two colored zones appear in the middle portion of the column, the upper one being pink and the lowed one being yellowish orange in color. After 40 ml. of the developing solution having flowed out, the column is pressed out with a glass rod, the pink portion is taken in a test-tube, about 3 ml. each of methanol and petroleum benzine is added, the mixture is shaken vigorously and about 3 ml. of water is added. Petroleum benzine layer containing the pigment is then separated. Being filled with CO_2 this layer is stored in a test-tube closed with a ground glass-stopper. A solution with E-value of 2.0 estimated in Pulfrich photometer (S47; 0.5 cm.) is adequate. X pigment is a kind of carotenoid, a petroleum benzine solution of which has two absorption maxima, namely at 445 and 469.5 mμ as shown in Fig. 1.

Fig. 1



Upon addition of antimony reagent a reddish purple color appears immediately and discolors rapidly.

PROCEDURE

1. *Saponification and extraction*

a g. of liver oil (usually 0.1 g.) is taken in a glass-stoppered test-tube (about 60 ml.) dissolved in 5 ml. of 5 % KOH in ethyl alcohol, heated at 75°C for 30 minutes in H_2, cooled with water and is dissolved in 10 ml. of benzene, H_2 being filled in a gas-space. After addition of 10 ml. of water, H being filled in a gas-space, it remains standing till the benzene layer separates completely. The latter being taken in a glass-stoppered centrifuge-tube (about 50 ml.) is washed twice with 10 ml. of 5 % KOH in 60 volume % of methanol and several times with 10 ml. of water, each time H_2 being filled. The clear benzene layer is taken quantitatively in a test-tube, evaporated completely, and the residue is dissolved in exactly *v* ml. (usually 5 ml.) of petroleum benzine.

2. *Chromatographic fractionation*

Two adsorption columns are prepared, one for main test and the second for blank test. As an adsorbens activated alumina "BL6" is used. The length of the column is 6–6.5 cm. and the velocity of the flow is 20–25 drops per minute. To the aspirating flask is attached a stop-cock by means of a rubber tube, through which the air is aspirated to regulate the rate of flow. *c* ml. (usually 1 ml.) of the test solution in petroleum benzine and 3 drops of the X pigment solution is allowed to pass through the column, 1 ml. of petroleum benzine is followed washing the wall of the tube, then 10 % solution of acetone in petroleum benzine is used for development. After about 10 ml. having flowed out, the flowing solution is collected quantitatively in a test-tube. After flow of about 30 ml. of the solution, the pigment layer comes to the position about 2-3 cm. below the surface of the column. The pigment layer is about 2 cm. in width. The upper part being thicker and sharper than the lower one, it is more convenient to estimate the position of vitamin D from the upper limit of the pigment layer. Having reached the upper limit to about 2.5 cm. above the lower end of the tube, the development is interrupted, vitamin D being completely collected in the tube. Intermixture of a small quantity of carotenoids has no effect as they are removed by the following treatment. Usually development with about 35–40 ml. of solvent is enough for the estimation of vitamin in liver oil, which takes usually 2.5–3 hours.

Remarks: The amount of vitamin A in the test solution should not exceed 50 micrograms, in order that the separation of D from A is complete, the intermediary zone free from vitamins being about 1.5-2.0 cm. in width.

3. Removal of the effect of intermingled carotenoids and steroids

The vitamin D fraction is evaporated completely by in a test-tube. In case of much non-saponifiable fraction after dissolution in a small quantity of methanol it is evaporated under reduced pressure. Being dissolved in 1 ml. of dioxane, 2 ml. of 10 % maleic anhydride is added. The one tube (main test) is heated at 80°C for 10 minutes and the second (blank test) to 100°C for 30 minutes. After cooling 10 ml. of 0.5 *N* KOH in ethyl alcohol is added, filled with hydrogen, placed for standing for 10 minutes, then 15 ml. of water being added. It is extraced with 5 ml. of petroleum ether three times. The collected extract is evaporated in vacuum completely. To secure complete evaporation 1 ml. of methanol is added, and after dissolution it is evaporated, this treatment being repeated once more. In case of existence of a small amount of methanol the color reaction is somewhat inhibited, so that the complete removal of methanol is absolutely necessary. It is dissolved in *m* ml. (usually 0.4 ml.) of chloroform.

Remarks:

(1) In case of high concentration of vitamin D, one chromatographic column is sufficient. Then the D fraction is made to a constant volume, and divided in two equal parts, one for the main test and the other for the blank test. Each tube is evaporated and treated with maleic anhydride. In case of high concentration of A and low concentration of D, D fractions of several chromatographs are collected together for treatment with maleic anhydride.

(2) The E value of carotene after treatment of maleic anhydride

at 80°C for 10 minutes reduces to about 70 %, and that of vitamin A to about 30 %, but after treatment at 100°C for 30 minutes the values remain practically the same as after treatment at 80°C for 10 minutes. The E value of ergosterol after treatment at 80°C for 10 minutes reduces to about 50 %, and that of cholesterol to about 30 %, but after treatment at 100°C for 30 minutes remains the same as after treatment at 80°C for 10 minutes. Therefore if the difference of both E values is taken for calculation, the effects of carotenoids and steroids are excluded.

4. Color reaction and determination

0.2 ml. of the above chloroform solution containing vitamin D not exceeding 10 μg. is pipetted directly into the 1 cm. cuvette, to which 3.0 ml. of antimony reagent is added in rapid stream. The cuvette is covered with a cover glass. E value is estimated in Pulfrich photometer (S 50) successively and the maximun E-value (usually in 3–4 minutes) is read. At the same time the room temperature is recorded. The blank E value rises gradually without reaching to a maximum. For calculation the blank value is taken at that time, when the maximum is reached in the main test.

The concentration of vitamin D is calculated after the following formula.

$$x=f\ (E-E_0)\times\frac{mv}{0.2\times ca}=5\ f\ (E-E_0)\frac{mv}{ac}\ \mu\text{g/g}$$

E=main value, E_0=blank value,

f=conversion factor.

In case $a=0.1,\ v=5,\ c=1,\ m=0.4$

$$x=100\ f\ (E-E_0)\ \mu\text{g/g}$$

$$=4{,}000\ f\ (E-E_0)\ \text{I. U./g}$$

f depends upon room temperature, the values of which are shown in Tab. I.

TABLE I.

Temperature (°C)	f
10	31
12	30
14	29
16	28
18	27
20	26
22	25
24	24
26	23
28	22
30	21

Remarks.

(1) Dioxane solution alone shows E value of about 0.015. after addition of antimony reagent but as $E-E_0$ is taken for calculation, the correction is not necessary.

(2) That there is in liver oil practically no other substance reacting with antimony reagent except vitamin D, which remains unchanged after heating at 80°C for 10 minutes and is completely destroyed by heating at 100° for 30 minutes is confirmed by the fact that the values estimated by this method agree fairly well with those estimated by animal assay (Tab. III).

Example:

Liver oil of Katsuwonus vagans Lesson.

$E=0.246$, $E_0=0.021$, $E-E_0=0.225$, temperature 22°C.

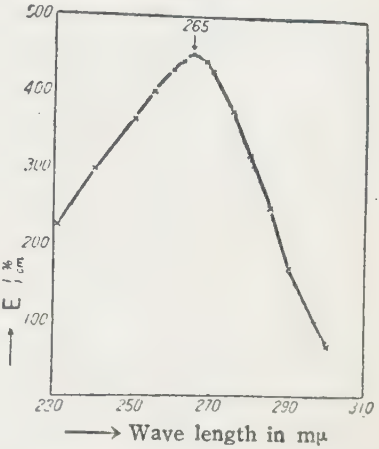
$x=100\times0.225\times25=562$ $\mu g/g=22{,}500$ I. U./g.

EXPERIMENTAL

1. *Absorption spectrum of calciferol solution.*

1.0 mg./100 ml. solution of pure calciferol (m.p. 115°) in ethyl alcohol measured in Beckman spectrophotometer is shown in Fig. 2. The absorption maximum lies at 265 mμ, at which $E^{1\%}_{1\,\mathrm{cm}}$ was 445.

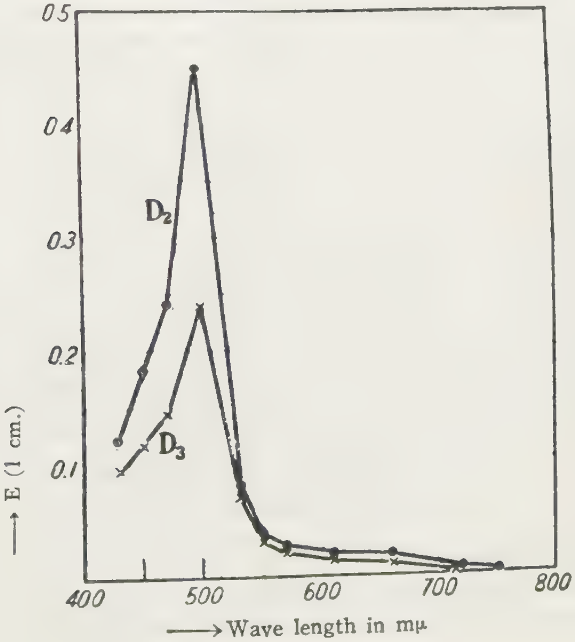
Fig. 2.



2. *Absorption spectrum of the color produced by antimony reagent*

To 15 μg of calciferol in 0.2 ml. of chloroform is added 3 ml. of

Fig. 3.

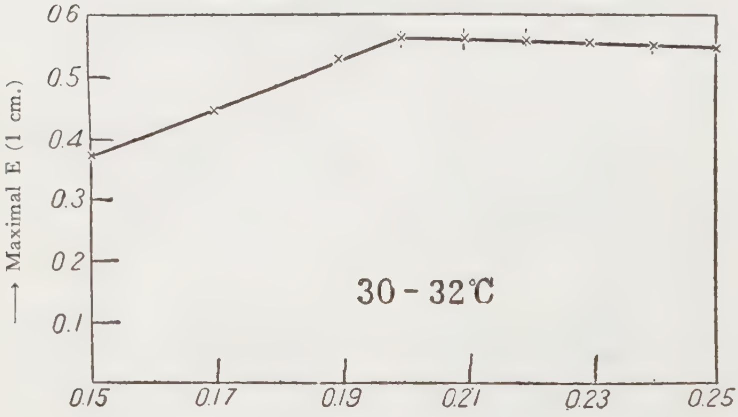


200 mg./ml. antimony trichloride solution. Yellowish orange color appeared immediately. After 6–11 minutes the E values (1 cm.) was determined in Pulfrich photometer. As shown in Fig. 3 the absorption maximum lies at about 500 mμ. The absorption curve of the 5.6 μg. of D_3 solution, prepared from liver oil, separated chromatographically and treated in the similar way is also shown in Fig. 3.

3. *Relation of E to concentration of $SbCl_3$*

To 10 μg. of calciferol in 0.2 ml. of chloroform is added 3.0 ml. of a solution of $SbCl_3$ in chloroform in various concentration. The maximal E values of each solution determined in Pulfrich photometer (S 50, 1 cm., 30—32°) are shown in Fig. 4. It is evident that the highest E value is obtained at the concentration of 200 mg./ml. of $SbCl_3$.

Fig. 4.

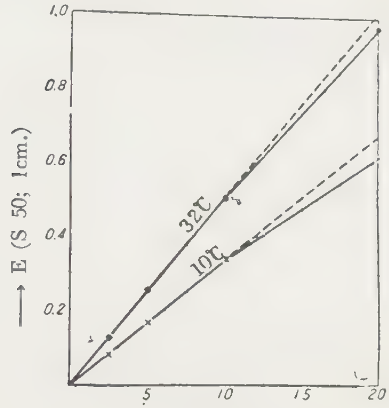


⟶ SbC_3l Concentration (g./ml.)

4. *E values and concentrations of calciferol*

The antimony reagent is added to various amount of calciferol. The E values change with time. The E value reaches its maximum in 2 minutes at 32°, whereas at 10° the maximum is eached in 4 minutes. The E value is remarkably higher at 32° than at 10°. The E values are proportional to concentrations of vitamin D, within 10 μg. of the vitamin as is shown in Fig. 5.

Fig. 5



⟶ Amount of vitamin D
(μg./0.2 ml.)

5. *Condensation of vitamin D, carotenoids and steroids with maleic anhydride*

To various amount of vitamin D_2, carotene, vitamin A, ergosterol and holesterol, respectively dissolved in 1 ml. of dioxane 2 ml. of 10 % maleic anhydride solution in dioxane is added, heated at x° for t minutes, mixed with 10 ml. of 0.5 N KOH in ethanol in hydrogen, then 15 ml. of water being added. It is extracted with 5 ml. of petroleum ether three time the gcas-space being filled with ${}_2$H or CO_2. After complete evaporation of petroleum ether, the residue is dissolved in chloroform and as described in the procedure, the color produced is determined photometrically.

TABLE II

Substance	Amount	x	t	E
Vitamin D	µg 10	°C 80	0 min.	0.137
			5	0.137
			10	0.137
			15	0.133
		100	0	0.137
			10	0.052
			20	0
Carotene	12	—	—	0.063
		80	10	0.043
		100	30	0.045
Vitamin A	20	—	—	0.207
		80	10	0.063
		100	30	0.057
Ergosterol	2,500	—	—	2.440
		80	10	1.079
		100	30	1.055
Cholesterol	2,500	—	—	0.310
		80	10	0.099
		100	30	0.097

As Table II shows, vitamin D_2 is unchanged by heating at 80° for 10 minutes and is completely destroyed by heating at 100° for 30 minutes. Carotenoids and steroids are considerably destroyed colorimetrically by heating at 80° for 10 minutes, but after heating at 100° for 30 minutes the destruction does not proceed further than by heating at 80° for 10 minutes.

6. Recovery of the added D_2 throughout the whole procedure

0.1 g. each of the liver oil of Theragra chalcogramma Pellas (1), containing 31,000 I. U. per g. of A and less than 100 I. U. of D_3 and that of Katsuwonus vogans Lesson (2), containing 8,800 I. U. per g. of A and 20,000 I. U. of D_3 are saponified and each non-saponifiable fraction is dissolved in 5 ml. of petroleum benzine, 1 ml. of (2) with or without addition of 5 ml. of (1) is treated as is described in the procedure. As the results in Tab. III shows, the added D_3 is practically completely recovered.

TABLE III

	E	E_0	$E-E_0$
(1)+(2)	0.256	0.058	0.198 (95%)
(2)	0.239	0.030	0.209

7. *Comparison with results of animal assay*

As is shown in Tab. IV, the values determined by the procedure described above agree well with those of animal assay, showing that the method is quite specific for vitamin D.

TABLE IV

Liver oil of	E	E_0	$E-E_0$	I. U./g	Animal assay
				$\times 10^3$	$\times 10^3$
Katsuwonug vogans A	0.402	0.092	0.310	32.8	Prophylactic:
	0.392	0.084	0.308	33.2	36.0
				Average	Therapeutic:
				33.0	33.0
„ B	0.246	0.021	0.225	22.5	21.88
	0.229	0.027	0.212	21.2	
	0.282	0.067	0.215	22.0	
				Average	
				21.9	
„ C	0.218	0.056	0.162	17.5	19—20
	0.225	0.048	0.177	18.7	
				Average	
				18.1	

SUMMARY

After through examination of the fundamental conditions of color reaction, chromatography and removal of the effects of intermingled carotenoids and steroids, we succeeded in determining chemically the vitamin D value of liver oil, the procedure of which was described in detail.

Of the fundamental conditions the following facts were confirmed by our studies.

The maximal absorption of the colored reaction product lies at about 500 mμ. E value is dependent of the concentration of antimony trichloride, 200 mg./ml. being the most adequate. After addition of the reagent the E value rises to a maximum and then decreasing gradually.

The time arriving to the maximum is dependent of the temperature. The maximum E values are propotional to the amount of D, within 10 μg. Using activated alumina "BL6" as an adsorption column and 10 % acetone in petroleum benzine as a developing solvent, the separation of A and D is practically complete, as long as the amount does not exceed 50 μg. As intermingled peroxide in solvent destroys vitamin D, it must be removed previously, if the reagent is not free from it.

A specific pigment "X" could be isolated from red pepper, which behaves chromatographically in the same way as vitamin A and it makes possible to observe the position of vitamin A. By the treatment of maleic anhydride in dioxane, vitamin D remains unchanged by heating at 80°C for 10 minutes but is destroyed completely by heating at 100°C for 30 minutes. Carotenoids and steroids are destroyed considerably by heating at 80°C for 10 minutes but the destruction does not go further after heating at 100°C for 30 minutes. The difference of the extinctions after heating at both conditions agree quite well with the extinction of added vitamin D alone. The added vitamin D is practically completely recovered throughout the whole treatment.

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A STUDY OF FERRITIN: ON THE LIBERATION OF IRON FROM FERRITIN

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It has been substantially proved by the recent works of Laufberger (1), Kuhn (2), Michaelis (3, 4, 5) Granick (3, 4, 6, 7, 8, 9), and Rothen (10) that an iron-protein compound called ferritin plays an important rôle in the metabolism of iron in the body. It is claimed by them that ferritin is a sort of storage of iron, from which iron is furnished as the demand occurs. Moreover, although the evidence was raised that the iron in ferritin is attached to a specific protein, apoferritin, in the form of ferric hydroxide micelles, the biologically interesting fact such as the liberation of iron from ferritin remains still obscure. In this regards Michaelis *et al.* (4) have only reported that iron is liberated from ferritin *in vitro* by the reducing action of sodium dithionite. The author attempted to investigate what sort of naturally occurring agents are concerned in this liberation.

At first, the results obtained by Michaelis *et al.* on the action of sodium dithionite was confirmed by the author, and, further, biologically important reductants, such as L-ascorbic acid and L-cysteine hydrochloride, were tested. As Tables II and III show, the iron in ferritin was gradually liberated by these substances. It seems to be true, therefore, that some reducing process is necessary to detach iron from ferritin. It is doubtful, however, how far do they play the actual rôle in process involved *in vivo.* Since in the body there are a lot of more active reducing activities due to various dehydrogenase systems, the author carried out many experiments in this concern. As well known, ferritin is contained in the large amount in liver and liver is a working factory in the iron metabolism. Using the liver brei of guinea pig, its action upon ferritin was observed. As shown in Tables IV, V, VI and VII, iron was liberated from ferritin when incubated with liver brei. But the liberation was inhibited markedly by sodium fluoride, monoiodoacetic acid, pteroylglutamic acid, only slightly by potassium cyanide in the concentration of *M*/1000 but not by malonic acid. Amoung these inhibitory effects, that of pteroylglutamic acid is most interesting, since it was reported by many investigators that pteroylglutamic acid is able to inhibit specifically the action of flavin containing enzymes, i. e., xanthine oxidase (11, 12, 13, 14), L-amino acid oxidase (12, 15, (16), D-amino acid oxidase (12, 14), diaphorase (12) and glycine oxidase (17). It might be true, therfore, that these enzymes may participate in the process of liberation. In fact, the author was able to confirm that the xanthine oxidase prepared from fresh milk cream and diaphorase preparation obtained from pig heart muscle are fairly active agents in this concern.

In view of these evidences, it may therefore be supposed that the enzymes playing the important rôle in the liberation of ferritin iron are either pyridinoprotein enzyme in cooperation with flavoprotein enzyme or flavoprotein enzyme alone. But the facts obtained by isolated substrate-enzyme systems, namely hypoxanthine-xanthine oxidase and reduced diphosphopyridinenucleotide-diaphorase systems, demonstrate clearly, as are seen in Table IX and X, that pyridinoprotein enzyme cannot attack ferritin directly but only in the presence of flavoprotein enzyme, the reduced form of which seems to be able to detach iron from ferritin. Subsequently, the question is raised which is the main agent involved in the liberation of iron from ferritin under the physiological circumstances. Considering the facts that the action of hypoxanthine-xanthine oxidase system upon ferritin is marked under anaerobic conditions, but not so much under aerobic, as is shown in Table IX, it might be assumed that diaphorase is more important in this case. Further, the author tested the action of acetylcholine upon ferritin *in vitro*, since Fukushima *et al.* (18) have recently reported that the plasma iron level increases by the action of acetylcholine *in vivo*. It was found as indicated in Table VIII that acetylcholine itself of even in the presence of liver brei has no apparent effect on ferritin.

EXPERIMENTAL

Ferritin employed was prepared from horse spleen by the method of Granick (6), recrystallized twice. The iron content was found to be in the range of 18.2–20.1 %.

Table I. shows the value of magnetic susceptibility of ferritin-iron determined by the Michaelis' defferential method (19) modified by the author (20). The deta represent the gram atomic susceptibility of iron (Xg-atom (Fe)) in units of 10^{-6} c. g. s. u. which are in accord with that obtained by Michaelis *et al.* (4). As is described below, the three-unpaired electrontype of ferric atom is characteristic of ferritin causing definite magnetic value which does not alter in the presence of many inorganic and organic substances. But when iron is set free from it, the change of magnetic value is noticeable, so that by following this change we can see how far iron is liberated from ferritin upon the treatment with agents biologically interesting. The data given in the following tables show the mean value of 2 or 3 experiments except in the case of diphosphopyridine nucleotide and diaphorase. In each case the special care was taken to avoid the contamination of iron in all reagents as possible as we can.

(*1*) *Effects of L-ascorbic acid and L-cysteine hydrochloride*

(a) Effect of L-ascorbic acid

Into the upper compartment of the apparatus 9.0 ml. of the ferritin solution was taken, which consists of 3.0 ml. of ferritin solution containing iron in the concentration of *M*/93.7, 3.0 ml. of *M*/10 veronal buffer (pH 7.0) and 4.0 ml. of redistilled water. To this 0.5

ml. of 5.0 % L-ascorbic acid solution was added. The end concentration of ferritin iron is *M*/330 and that of L-ascorbic acid 0.264 g./dl. The measurement was carried out at the room temperature (24°C). The results are shown in Table II.

As will be seen fron the table, the value of Xg-atom (Fe) increases with time. This means that iron is gradually liberated by L-ascorbic acid.

(b) Effect of L-cysteine hydrochloride

Experimental procedures are quite the same as in (a) except that L-cysteine hydrochloride was used instead of L-ascorbic acid and the end concentration of ferritin iron is *M*/374 and that of L-cysteine hydrochloride 0.20 g./dl. Since cysteine solution is acidic, it was neutralized with alkali just before use. As Table III shows, the value of Xg-atom (Fe) increases with time but not markedly in this case.

In this regards Michaelis *et al.* (4) reported that iron is detached from ferritin by the action of sodium dithionite. The author was also able to confirms this fact.

(2) *Experiments employing guinea pig liver brei*

Liver removed from guinea pig sacrificed be decapitation and bleeding was homogenized with double amount of ice-cold *M*/10 veronal buffer (pH 7.0) using the Potter and Elvehjem's homogenizer (21). To 3.0 ml. of this brei was added 3.0 ml. of ferritin solution and 4.0 ml. of redistilled water. When the effect of inhibitors for dehydrogenase is to be investigated, it is added in solution in place of water. In these cases the control experiments should be done on the solution containing only liver brei and inhibitor and the magnetic value found should be substracted from that of the main system. Thus we can obtain the value attached to ferritin and its altered form.

(a) Effects of sodium fluoride and monoiodoacetic acid

Into the system above mentioned, sodium fluoride and monoiodoacetic acid were added in the concentrations of *M*/100 and *M*/200 respectively and incubated over a period of 2 hours at 38.0° C. The end concentration of ferritin-iron is *M*/195 in these cases. Table IV shows the results obtained under these conditions.

It is clearly demonstrated that liver brei has the power to liberate iron from ferritin under the experimental condition given above. That this is due to the enzymes in liver brei can be well acknowledged by the fact that it is abolished by heating over a period of 15 minutes at 80°C. It was also observed that the change of the magnetic value is caused by the free iron atom by the fact that ferric iron added to liver brei in the concentration of *M*/500- *M*/1000 gave the corresponding value of 3850×10^{-6} c. g. s. u.

Additional evidence was also raised by analysing the iron and nitrogen content of the ferritin recovered after attack of liver brei on it.

	Fe content	N content
The original ferritin	20.0 %	10.2 %
The recovered ferritin	16.8 %	10.5 %

This result coincides with the magnitude of the change of magnetic susceptibility observed.

(b) Effect of potassium cyanide.

Potassium cyanide was added in the concentration of *M*/500 or *M*/1000, and the reaction flasks were incubated over a period of 2 hours at 38°C.

The end concentration of ferritin-iron was *M*/189. As shown in Table V, potassium cyanide inhibits only slightly the liberation of iron from ferritin.

(c) Effect of malonic acid.

Malonic acid brought to pH 7.0 with sodium hydroxide before use was added to in the concentration of *M*/100. The end concentration of ferritin iron was $M/155$. The reaction system was kept at 38°C over a period of 2 hours.

The results are reported in Table VI. It seems malonic acid which is assumed to be an inhibitor for succinic dehydrogenase has no apparent effect.

(d) Effect of pteroylglutamic acid (PGA)

PGA previously brought to pH 7.0 with sodium hydroxide was added in the concentration of $200\gamma/10$ ml. or $400\gamma/10$ ml. into the reaction system which was kept at 38°C during 2 hours. The end concentration of ferritin-iron was $M/133$. The results obtained are shown in Table VII.

(e) Effect of acetylcholine

The concentration of acetylcholine and ferritin-iron were 20 mg./dl. and $M/130$ respectively. The reaction flask was kept at 38°C during 2 hours. Table VIII shows the results obtained.

(3) *Experiments by means of some isolated substrate-enzyme system*

(a) Hypoxanthine-xanthine oxidase system

Xanthine oxidase was prepared from fresh milk cream according to Ball (22) and hypoxanthine was used as its substrate, which was dissolved in $M/25$ veronal buffer (pH 7.0) in the concentration of $M/80$. The activity of this enzyme preparation was fairly high, the decolorization time being 50 seconds at 38°C in the system of hypoxanthine solution 2.8 ml. $M/5000$ methylene blue solution 1.0 ml. redistilled water 0.9 ml. and xanthine oxidase solution 0.3 ml.

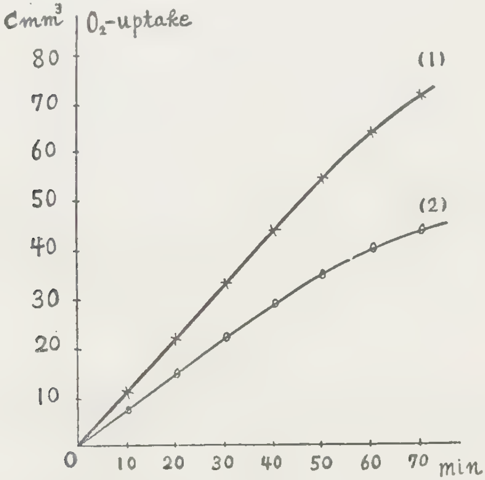
Employing the same xanthine oxidase solution and ferritin solution (the concentration of iron was $M/200$), the results reported in Table IX were obtained.

Fig. 1 shown the inhibitory effect of PGA on the same xanthine oxidase preparation. Oxygen uptake was measured manometrically by the Warburg's apparatus. Control vessel contains in main chamber M/X veronal buffer (pH 7.0) 1.0 ml. +xanthine oxidase 0.4

ml. +redistilled water 1.3 ml. in side bulb *M*/20 hypoxanthine dissolved in *N*/20 NaOH 0.3 ml. and in the center compartment 10% KOH 0.2 ml. The PGA-containing vessel contains in main chamber *M*/10 veronal buffer (pH 7.0) 1.0 ml.+xanthine oxidase 0.4 ml.+0.4 mg./ml. PGA 0.3 ml.+redistilled water 1.0 ml. in side bulb *M*/20 hypoxanthine dissolved in *N*/20 NaOH 0.3 ml. and in the center compartment 10% KOH 0.2 ml. Both vessels were kept at 37°C. shaken 120 times per minute and after 15 minutes hypoxanthine was poured into the main chamber. Gas space contains air.

Fig. 1

Inhibitory Effect of Pteroylglutamic Acid on Xanthine Oxidase



(1) Main chamber: *M*/10 veronal buffer (pH 7.0) 1.0 ml. xanthine oxidase 0.4 ml. redistill. water 1.3 ml. Side bulb: *M*/20 hypoxanthine in *N*/20 NaOH 0.3 ml. Center compartment: 10% KOH 0.2 ml.

(2) Main chamber: *M*/10 veronal buffer (pH 7.0) 1.0 ml. xanthine oxidase 0.4 ml. PGA. (0.4 mg./ml.) 0.3 ml. redistill water 1.0 ml. Side bulb: *M*/2) hypoxanthine in *N*/20 NaOH 0.3 ml. Center compartment: 10% KOH 0.2 ml.

Gas space contains air. Temperature: 37.0°C.

As is illustrated in Fig. 1, PGA inhibits markedly the action of xanthine oxidase

(b) Diphosphopyridine nucleotide (DPN)+Diaphorase.

DPN was prepared from pressed baker's yeast according to Williamson and Green (23), converted into reduced from (DPN-H_2) by the method of Green and Dewan (24) and brought to pH 8.5 just before use. Its purity was determined as 60.1 % by measuring its total-P content. Diaphorase was prepared from pig heart muscle by the method of Euler and Hellstroem (25), dissolved in $N/10$ ammonium hydroxide solution and brought to pH 8.5 with diluted hydrochloric acid just before use. The measurement was done on the following system: $M/10$ veronal buffer (pH 8.5) 1.0 ml.+DPN-H_2 solution 2.0 ml.+diaphorase solution 1.0 ml.+$M/5000$ methylene blue solution.

The activity of enzyme can be well judged from the fact that methylene blue bleached after 2.5 minutes kept at 38°C. Using the same diaphorase solution, various reacting systems were prepared as given in Table X and their magnetic values were determined as usual. The end concentration of ferritin-iron was $M/980$ in these cases. The results are given in Table X.

TABLE I

The Value of Magnetic Susceptibility of Ferritin

	χg. atom. (Fe) ($\times 10^{-6}$ c. g. s. u.)
Ferritin-iron Concentration: $M/312.3$	6000 6080 5950

Magnetic Measurement at 24.0°C.

TABLE II

Change of Magnetic Susceptibility of Ferritin-Iron on the Action of L-Ascorbic Acid

Time (minutes)	0	10	140	340
χ g. -atom. (Fe) ($\times 10^{-6}$ c. g. s. u.)	6080	6280	6430	6600

Magnetic Measurement at 24.0°C.

TABLE III

Change of Magnetic Susceptibility of Ferritin-Iron on the Action of L-Cysteine Hydrochloride

Time (minutes)	0	20	290	1440
χ g. -atom. (Fe) ($\times 10^{-6}$ c. g. s. u.)	6100	5630	6200	6500

Magnetic Measurement at 24.0°C.

TABLE IV

Effects of Sodium Fluoride and Monoiodoacetic Acid on the Liberation of Ferritin-Iron caused by the Action of Liver Brei

	Ferritin	Ferritin NaF	Ferritin CH_2ICOOH	Ferritin Liver-Brei	Ferritin Liver-Brei NaF	Ferritin Liver-Brei CH_2ICOOH
χ g. -atom. (Fe) ($\times 10^{-6}$ c. g. s. u.)	6070	6100	6120	5480	5980	6080

Magnetic Measurement at 11.0°C.

TABLE V

Effects of Potassium Cyanide on the Liberation of Ferritin-Iron caused by the Action of Liver Brei

	Ferritin	Ferritin KCN		Ferritin Liver-Brei	Ferritin Liver Brei KCN	
		$(M/500)$	$(M/1000)$		$(M/500)$	$(M/1000)$
χ g. -atom. (Fe) ($\times 10^{-6}$ c. g. s. u.)	5950	6000	5970	5240	5800	5300

Magnetic Measurement at 11.5°C.

TABLE VI

Effects of Malonic Acid on the Liberation of Ferritin-Iron caused by the Action of Liver Brei

	Ferritin	Ferritin Malonic Acid	Ferritin Liver-Brei	Ferritin Liver-Brei Malonic Acid
χ g. -atom. (Fe) ($\times 10^{-6}$ c. g. s. u.)	6080	6000	5410	5290

Magnetic Measurement at 11.0°C.

TABLE VII

Effects of Pteroylglutamic Acid on the Liberation of Ferritin-Iron caused by the Action of Liver Brei

	Ferritin	Ferritin PGA. (400γ)	Ferritin Liver-Brei	Ferritin Liver-Brei PGA. (200γ)	Ferritin Liver-Brei PGA. (400γ)
χg. -atom. (Fe) ($\times 10^{-6}$ c. g. s. u.)	6080	6100	5410	5670	5900

Magnetic Measurement at 11.0°C.

TABLE VIII

Effects of Acetylcholine on the Liberation of Ferritin-Iron caused by the Action of Liver Brei

	Ferritin	Ferritin Acetylcholine	Ferritin Liver-Brei	Ferritin Liver-Brei Acetylcholine
χg. -atom. (Fe) ($\times 10^{-6}$ c. g. s. u.)	6090	6020	5170	5200

Magnetic Measurement at 11.0°C.

TABLE IX

Action of Hypoxanthine-Xanthine Oxidase System on Ferritin

Reaction System (Totol Volume) 9.0 ml.	Ferritin 3.0 *M*/10 Veronal-Buffer (pH 7.0) 2.0 Redistill. Water 4.0	Ferritin 3.0 *M*/80 Hypoxanthine in M/25 Veronal-Buffer (pH 7.0) 5.0 Xanthine Oxidase 0.5 Redistill. Water 0.5	Ferritin 3.0 *M*/80 Hypoxanthine in *M*/25 Veronal-Buffer (pH 7.0) 5.0 *M*/5000 Methylene Blue 0.5 Xanthine Oxidase 0.5	Ferritin 3.0 *M*/80 Hypoxanthine in *M*/25 Veronal-Buffer (pH 7.0) 5.0 Xanthine Oxidase 0.5 Redistill. Water 0.5	Ferritin 3.0 *M*/80 Hypoxanthine in *M*/25 Veronal-Buffer (pH 7.0) 5.0 Redistill. Water 1.0
Condition	38°C 1 hr. aerob	38°C 1 hr. anaerob	38°C 1 hr. anaerob	38°C 1 hr. aerob	38°C 1 hr. aerob
χg. -atom. (Fe) ($\times 10^{-6}$ c. g. s. u.)	6000	4520	4100	5750	6020

Magnetic Measurement at 7.0°C.

TABLE X

Actions of Reduced Diphosphopyridine Nucleotide and Diaphorase on Ferritin

Reaction Systems (Total Volume) 10.0 ml.	Ferritin 2.0 *M*/10 Veronal-Buffer (pH 8.5) 2.0 Redistill. Water 6.0	Ferritin 2.0 *M*/10 Veronal-Buffer (pH 8.5) 2.0 DPN. 4.0 Redistill. Water 2.0	Ferritin 2.0 *M*/10 Veronal-Buffer (pH 8.5) 2.0 DPN. 4.0 Diaphorase 2.0		Ferritin 2.0 *M*/10 Veronal Buffer (pH 8.5) 1.5 PGA.* 0.5 DPN. 4.0 Diaphorase 2.0
Conditions	38°C 1 hr.	38°C 1 hr.	6°C 10 min.	38°C 1 hr.	33°C 1 hr.
χg. -atom. (Fe) ($\times 10^{-6}$ c. g. s. u.	5950	6200	5780	4900	5700

(*): The Concentration of PGA: 20.0 mg./dl.
Magnetic Measurement at, 6.0°C.

SUMMARY

(1) L-ascorbic acid and L-cysteine hydrochloride are able to liberate iron from ferritin *in vitro.*

(2) The liberation of iron from ferritin is performed by some enzymic process in liver which is inhibited markedly by sodium fluoride, monoiodoacetic acid, pteroylglutamic acid, but slightly by potassium cyanide in the concentration of *M*/1000 and not by malonic acid.

(3) Hypoxanthine-xanthine oxidase system is able to liberate iron from ferritin under anaerobic circumstances, especially in the presence of methylene blue, but only slightly under aerobic conditions. Reduced diphospyridine nucleotide-diaphorase system exhibits the iron-liberating action upon ferritin which is inhibited by pteroylglutamic acid.

(4) These evidences seem to demonstrate that diaphorase participates chiefly in the liberation of the ferritin iron under the physiological circumstances.

(5) It was proved that acetylcholine itself or even in the presence of liver brei has no apparent effect on ferritin.

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